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A Laboratory Study in Support of the Pilot Demonstration of a Biological Soil Slurry Reactor

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13. ABSTRACT (MAXIMUM 200 WORDS)

The research conducted in this project demonstrated that TNT can be removed effectively by native soil bacteria via a co-metabolic process. The aerobic bacterial consortium developed from the TNT-contaminated soil successfully removed TNT in liquid cultures. Various co-substrates (glucose, succinate, acetate, malate, citrate, and molasses) were studied and evaluated. Among these, molasses was identified as the best substrate for TNT removal. Aerobic optimization studies revealed that a pH of 7.0, a temperature of 22-24°C, and an ammonium concentration of 0.5 g/L are optimal for degradation of TNT by the bacterial consortium. The successful operation of an aerobic/anoxic laboratory-scale soil slurry reactor showed that contaminated soil could be treated. Batch treatment resulted in 100% removal of TNT within 21 days. Semicontinuous treatment also resulted in complete degradation of TNT. Apart from removing TNT, the slurry reactor also removed trinitrobenzene, 2,4-dinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine. Radiolabeling studies on the reactor biomass showed that 23% of [^{14}C]TNT was mineralized, 27% was converted to biomass, and 8% was adsorbed to the soil. The rest of the [^{14}C]TNT was accounted for as TNT metabolites; the majority metabolite (23%) was identified as 2,3-butanediol. Increasing the frequency of soil addition from one time to two or three times a week did not affect the TNT removal rates.

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Notation

APHA	American Public Health Association
°C	degrees Celsius
CFU	colony-forming units
cm	centimeter
cpm	counts per minute
DNT	2,4-dinitrotoluene
g	gram
gal	gallon
GC/MS	gas chromatography/mass spectrometry
h	hour
HMX	octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine
HPLC	high-performance liquid chromatography
kg	kilogram
L	liter
m	meter
mg	milligram
min	minute
mL	milliliter
mM	millimolar
mm	millimeter
µCi	microcurie
µL	microliter
µm	micrometer
N	normal
nm	nanometer
psi	pounds per square inch
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine
RLRW	aerobic bacterial consortium enriched from soil at Joliet Army Ammunition Plant
rpm	revolutions per minute
s	second
TCA	trichloroacetic acid
TNB	trinitrobenzene
TNT	2,4,6-trinitrotoluene
TSA	Tryptic Soy Agar
USAEC	U.S. Army Environmental Center (formerly USATHAMA)
USATHAMA	U.S. Army Toxic and Hazardous Materials Agency (now USAEC)
V/V	volume/volume
W/V	weight/volume

A Laboratory Study in Support of the Pilot Demonstration of a Biological Soil Slurry Reactor

Summary

Among the pollutants unique to the military and associated agencies are those arising from the manufacture, handling, and demilitarization of munitions. 2,4,6-Trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine are widely used for military purposes. Disposal of the explosives during manufacturing and testing has resulted in damage to water and soil ecosystems. Remediation of explosives-contaminated soils and water and disposal of explosives are two important applications in which biological degradation of explosives may provide a safe and cost-effective approach. The research conducted in this project demonstrated that TNT can be removed effectively by native soil bacteria.

The bacterial consortium developed from the TNT-contaminated soil successfully removed TNT in liquid cultures within a short incubation period of 12 h. TNT removal is a co-metabolic process and needs a co-substrate such as succinate. Various co-substrates (glucose, succinate, acetate, malate, citrate, and molasses) were studied and evaluated. Among these, molasses was identified as the best substrate for TNT removal in large-scale treatment.

The aerobic optimization studies revealed that a pH of 7.0, a temperature of 22-24°C, and an ammonium concentration of 0.5 g/L are optimal for degradation of TNT by the bacterial consortium. The radiolabeling study on the bacterial consortium indicated that 3% of TNT was mineralized to $^{14}CO_2$, while 8-13% of TNT was converted to biomass and the rest to TNT metabolites. The mass balance was reasonably accurate. TNT removal by growing cells, resting cells, and cell-free extracts was also studied. The main TNT metabolites identified were 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene.

Four *Pseudomonas* spp. were isolated from the bacterial consortium. They were *Ps. acidovorans*, *Ps. fluorescens*, *Ps. mendocina*, and *Ps. aeruginosa*. The experiments conducted with individual *Pseudomonas* spp. suggested that each species has a specific function in the consortium.

The successful operation of an aerobic/anoxic laboratory-scale soil slurry reactor showed that the contaminated soil could be treated in batches or semicontinuously. Batch treatment resulted in 100% removal of TNT within 21 days. Semicontinuous treatment also resulted in complete degradation of TNT. Apart from removing TNT, the slurry reactor also removed trinitrobenzene, 2,4-dinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro- 1,3,5,7-tetranitro-1,3,5,7-tetraazocine. Radiolabeling studies on the reactor biomass showed that 23% of [^{14}C]TNT was mineralized, 27% was converted to biomass, and 8% was adsorbed to the soil. The rest of the

[^{14}C]TNT was accounted for as TNT metabolites, including a metabolite identified as 2,3-butanediol. Increasing the frequency of soil addition from one time to two or three times a week did not affect the TNT removal rates. However, the slurry reactor showed signs of stress, with acidic conditions and low oxygen uptake rates. The soil slurry reactor also maintained the bacterial population fairly well, needing only 0.3% molasses as a co-substrate.

1   Introduction

2,4,6-Trinitrotoluene (TNT) is a primary military explosive that was widely used in the past because of its low melting point; its stability; its low sensitivity to impact, friction, and high temperature; and its relative safe methods of manufacture (Urbanski 1984). TNT exists as odorless orthorhombic crystals or as yellow monoclinic needles. Disposal of explosives during manufacturing and testing has resulted in extensive contamination of soil (Klausmeier et al. 1973). The manufacture of explosives and the cleaning and repacking of old munitions require large quantities of water. The waste water from munitions processing is contaminated with TNT. For years this water was discarded outside manufacturing facilities, on the ground or in lagoons that leached explosives into soil, groundwater, rivers, and lakes (Pereira et al. 1979). Concerns about the environmental fate of TNT residues have intensified because the recent vegetation of contaminated plots could allow TNT, TNT metabolites, and plant-produced TNT intermediates to be introduced into the food chain (Harvey et al. 1990).

Studies sponsored by the U.S. Army Toxic and Hazardous Materials Agency (USATHAMA) (now the U.S. Army Environmental Center [USAEC]) have explored both composting and land farming techniques and have validated the concept of biologically decontaminating TNT-laden soils by using composting. Composting of explosives has proven to be effective, and half-lives for the breakdown of TNT have ranged from 7 to 22 days (Weston 1989). The disadvantage of composting is that it requires large quantities of additives (e.g., straw, animal feed); only a small fraction of the total volume composted is contaminated soil. The current method of remediation for explosives-contaminated soils is incineration. This is a costly, energy intensive process that destroys much of the soil, leaving ash as the primary residue. Funk et al. (1993) demonstrated an aerobic-anaerobic sequencing system for the bioremediation of TNT-contaminated soil. However, they showed poor mineralization of TNT; most of the TNT was converted to TNT metabolites.

Biological removal of explosives is also feasible. The purpose of this investigation was to determine whether native soil bacteria isolated from TNT-contaminated soil would grow in the presence of a highly toxic contaminant like TNT, whether they would degrade the TNT, and whether a biological reactor system could demonstrate, at the laboratory scale, potential as a biological treatment process.

The main objectives of this study were the following:

1. To study the ability of native soil bacteria from the contaminated site to degrade TNT and other explosives

2. To determine the optimal conditions of pH, temperature, and nitrogen concentration for maximum TNT degradation

3. To identify an inexpensive carbon source for TNT metabolism

4. To evaluate the ability of the soil slurry reactor technology to remove TNT from the contaminated soil

5. To identify the operating conditions for the pilot-scale treatment system

2　Review of Scientific Background

2.1　Aerobic Degradation of TNT

Initial reports of microbial degradation of TNT were made by Rejovskaya (Spanggord et al. 1980), who observed the biodegradation of TNT initially present at 5 mg/L. Chambers et al. (1963) reported that a bacterial culture adapted to metabolize phenol also oxidized TNT. Bringmann and Kuehn (Spanggord et al. 1980) observed 99.6% transformation of TNT after passage through a two-stage reactor. The first stage was aerated and contained an *Azotobacter* sp., while the second stage contained conventional activated-sludge microflora. Osmon and Klausmeier (1972) provided strong evidence that TNT transformation is a co-metabolic process. They observed that in cultures containing TNT (100 mg/L), mineral salts, and yeast extract, more than 99% of the TNT was removed after six days of incubation. On the other hand, no TNT disappearance was observed in flasks without the yeast extract. Nay et al. (1974) observed that TNT waste could be biologically treated in combination with domestic waste.

Three *Pseudomonas*-like organisms were shown to metabolize TNT (Won et al. 1974). The oxidation capability varied with each organism. The nitrate-reducing isolate degraded TNT most effectively, while the isolate that neither reduced nitrate nor formed indole was the least effective. The O_2 consumption by the nitrate-reducing isolate, inclubated for four weeks with TNT, was 23% greater than the endogenous uptake. The addition of glucose or a nitrogenous substance accelerated the TNT degradation. Degradation of TNT occurred most rapidly in cultures of the nitrate-reducing isolate supplemented with 0.5% yeast extract. The separation of TNT metabolites yielded 2,2',6,6'-tetranitro-4-azoxytoluene, 2,2',4,4'-tetranitro-6-azoxytoluene, and diaminonitrotoluene. After depletion of TNT (24 h) the azoxy compounds were degraded gradually, approaching complete disappearance at 96 h. However, the organisms were unable to oxidize the metabolite diaminonitrotoluene during the 96-h observation period.

Traxler et al. (1974) isolated from various sources a number of gram-negative bacteria that were capable of using TNT as a sole source of carbon and energy. They also determined that yeast extract (100 μg/mL) stimulated TNT metabolism. They found that 62% of the initial TNT was removed within 24 h from a medium supplemented with yeast extract. Radiolabeling studies indicated that a very low percentage of the ^{14}C initially added as [^{14}C]TNT was converted to $^{14}CO_2$ (0.3-1.2%). Washed cells effectively removed TNT from the medium with or without organic nutrients. After 18 h, 94% of the TNT was removed by a cell mass of 14 mg/mL. At lower concentrations, removal was poor (Traxler 1975).

Parrish (1977) described the results obtained from the screening of 190 cultures of fungi for the ability to transform TNT. Of the 190 organisms, 183 were able to transform TNT in a basal medium containing 0.5% glucose at a temperature of 29°C. Studies with radiolabeled TNT gave no evidence for cleavage of the carbon skeleton.

Naumova et al. (1979) isolated from soil polluted with industrial waste a *Ps. denitrificans* strain that transformed TNT concentrations as high as 200 mg/L to reduced metabolites within four days. Amerkhanova and Naumova (1978) studied microbial degradation of TNT by using *Ps. denitrificans* and *Escherichia coli*, with TNT as the only source of nitrogen and carbon for growth. The nitrogen of TNT was less accessible to *Ps. denitrificans* than to *E. coli*. The carbon of TNT was not used by *E. coli* and was barely accessible to *Ps. denitrificans*. Decomposition of TNT was enhanced by the addition of yeast extract to the medium.

Carpenter et al. (1978) investigated the fate of radiolabeled TNT in an activated-sludge system. No significant $^{14}CO_2$ was formed despite the absence of [^{14}C]TNT in the aerated reactor after three to five days. The radioactivity was found in ^{14}C-labeled transformation products that were evenly distributed between the floc and supernatant. Evidence indicated that TNT transformation products formed polymeric conjugates with lipids, fatty acids, and proteins. No evidence was found to indicate that the TNT aromatic nucleus was cleaved.

In a three-year investigation of a 3,000-gal pilot-scale oxidation ditch treatment system, Hoffsommer et al. (1978) found that the most efficient transformation occurred with activated-sludge microbes and supplemental nutrients. Less than 0.4% of the added [^{14}C]TNT was converted to $^{14}CO_2$, with the remainder being distributed between the solid (bacterial floc) and aqueous phases.

2.2   Bioremediation of TNT

Klausmeier and Jamison (1982) examined the composting of solid TNT. During a 74-day composting cycle, the TNT concentration decreased from 1% to 0.002%. Kaplan and Kaplan (1982) emphasized that the transformations of TNT are the same in thermophilic as in mesophilic systems. Kaplan and Kaplan (1983) also found that unreactive azylnitro groups became more reactive when they were microbially reduced to the corresponding azylamine. In addition, they found that some microbial transformation products were active breeding species. A significant proportion of the [^{14}C]TNT in compost systems was bound and not extractable with polar or nonpolar solvents. Recently Williams et al. (1992) studied the composting of explosives- and propellant-contaminated soils under thermophilic and mesophilic conditions. They found significant removal of TNT and its intermediates under both conditions.

The persistence of TNT in waste disposal lagoons was examined by Spanggord et al. (1983). The half-life of TNT with respect to biotransformation was calculated to be 69 days. Kaplan et al. (1984) observed that TNT transformation in soil proceeded at the highest rate at 0.1% TNT and was progressively slower at 1.0% and 10.0% TNT. Their results indicated that greater concentrations of TNT, smaller numbers of microbes, colder temperatures, and dryer soils all extend the period when TNT will remain unchanged.

A semicontinuous activated-sludge treatment system was used to treat munitions waste water by Bell et al. (1984), who found that no significant removal of TNT occurred under anoxic conditions at concentrations below 5 mg/L. Land application of TNT was believed to pose a threat to groundwaters and soils because of the toxicity of intermediate and parent compounds (Greene et al. 1985).

2.3   Anaerobic Degradation of TNT

Under anaerobic conditions a sulfate-reducing bacterium, *Desulfovibrio* sp. (B strain), reduced and reductively deaminated TNT to toluene (Boopathy and Kulpa 1992; Boopathy et al. 1993). Similar work on sulfate reducers has also been reported by Preuss et al. (1993). Biotransformation of nitroaromatic compounds by methanogenic bacteria was reported by Gorontzy et al. (1993). Funk et al. (1995) recently demonstrated anaerobic degradation of TNT at the laboratory and pilot scales.

3   Experimental Approach

3.1   Microorganisms

The TNT-contaminated soil samples were collected at the Joliet Army Ammunition Plant, Joliet, Illinois. The aerobic bacterial consortium, RLRW, was enriched from this soil sample. The enrichment cultures showing growth were transferred to fresh medium. After five transfers, the cultures were plated for isolation, and the isolates were tested for their ability to degrade TNT.

3.2   Growth of the Consortium

The RLRW consortium was cultivated in Stanier's medium (Stanier et al. 1966). Succinate was added at 0.5% (W/V) and TNT at 100 mg/L. To confirm that TNT was degraded, the RLRW consortium was grown in a heterotrophic medium consisting of the following components: K_2HPO_4 (7.0 g/L), KH_2PO_4 (3.0 g/L), $MgSO_4$ (0.10 g/L), NaCl (0.1 g/L), $(NH_4)_2SO_4$ (0.25 g/L), peptone (0.5 g/L), yeast extract (0.10 g/L), succinate (5 g/L), and TNT (100 mg/L). All culture flasks were incubated at 150 rpm on a gyratory shaker with a stroke length of 1.5 cm, at ambient temperature (22-24°C).

3.3   Co-metabolic Screening Study

To develop a (preferably inexpensive) co-substrate for field implementation, various co-substrates were screened with the RLRW bacterial consortium. For co-metabolic studies, the heterotrophic medium described in Section 3.2 was used, with the carbon source replaced with reagent-grade succinate, malic acid, acetate, sucrose, glucose, citrate, and molasses. All carbon sources except molasses were added at 0.5% (W/V). Molasses was added at concentrations of 0.1, 0.2, or 0.3% (W/V). For each carbon source, duplicate culture flasks were incubated. The experiment was repeated. Growth and TNT concentration were monitored every day. The cultures were incubated at 150 rpm on a gyratory shaker. A pregrown midlog-phase culture was used as inoculum (1%). The control flasks were heat inactivated by autoclaving after the inoculation of bacteria.

3.4   Effect of Temperature on the Bacterial Consortium

The RLRW bacterial consortium was incubated at room temperature (22-24°C), 25°C, 30°C, and 37°C in the heterotrophic medium described in Section 3.2. Growth and TNT concentration were monitored daily. For each temperature, duplicate culture flasks were

maintained. The cultures were incubated on a gyratory shaker set at 150 rpm. A pregrown midlog-phase culture was used as inoculum (1%). The optical density of the inoculum at 600 nm was 0.92. The bacteria in the control flasks were killed by autoclaving after inoculation.

3.5 Optimal pH for the Bacterial Consortium

To determine the optimal pH for growth of the RLRW bacterial consortium, the cultures were maintained at various pHs in the heterotrophic medium described in Section 3.2. The pH of the culture medium was adjusted by using HCl (1 N) or NaOH (1 N). The initial pH values used for the study were 6.0, 6.5, 7.0, 7.5, and 8.0. Growth and TNT concentration were monitored daily. Duplicate culture samples were maintained for each pH level studied. The cultures were incubated at room temperature on a gyratory shaker set at 150 rpm. A pregrown midlog-phase culture was used as inoculum (1%). The optical density of the inoculum at 600 nm was 0.90. The bacteria in the control flasks were heat inactivated by autoclaving after inoculation. The pH was monitored throughout the experiment.

3.6 Optimal Nitrogen Concentration for the RLRW Bacterial Consortium

Nitrogen is required for microbial growth. The inorganic form of nitrogen, reagent-grade ammonium chloride (NH_4Cl), was used as a supplemental nitrogen source in the heterotrophic medium described in Section 3.2 at concentrations of 0.1, 0.25, 0.5, and 1.0 g/L. One set of cultures had no supplemental source of inorganic nitrogen. Heat-inactivated cells served as the control. A pregrown midlog-phase culture used as inoculum (1%) had an optical density of 0.95 at 600 nm. For each nitrogen concentration duplicate flasks were maintained, and the experiment was repeated. Growth and TNT concentration were monitored daily. The cultures were incubated at room temperature on a gyratory shaker at 150 rpm.

3.7 Effect of Nitrogen-Reducing (Anoxic) Conditions on TNT Metabolism

To determine whether the RLRW bacterial consortium could metabolize TNT under anoxic conditions, using nitrate as electron acceptor, the heterotrophic medium described in Section 3.2 was prepared and dispensed into duplicate 100-mL bottles. The bottles were sealed with butyl rubber stoppers and crimped aluminum seals. The bottles were made anoxic by using an anaerobic gassing manifold. The head space of the culture was filled with 100% N_2 at 10 psi. In all of the bottles, sodium nitrate (20 mM) was used as the electron acceptor, and succinate (5 g/L) was used as carbon source. Heat-inactivated cells served as the control. An aerobically pregrown midlog-phase culture served as inoculum (5.0% by volume). The optical density of the inoculum was 0.90 at 600 nm. Growth and TNT concentration were followed daily. Growth was compared to

that of the aerobic cultures. The cultures were incubated at room temperature in a gyratory shaker set at 150 rpm.

3.8   TNT Metabolism in a Semicontinuous Culture

The RLRW bacterial consortium was cultured for 30 days in 100-mL flasks in the heterotrophic medium described in Section 3.2, with succinate (5 g/L) as the main carbon source. Every week TNT (50 mg/L) and succinate (0.2%) were added. Growth and TNT concentration were monitored continuously. Pregrown midlog-phase cultures (1%) served as inoculum. Cultures were incubated at room temperature in a gyratory shaker set at 150 rpm. Heat-inactivated cells served as the control.

3.9   Radiolabeling Study

Radiolabeled TNT (uniformly labeled on the ring with ^{14}C) (Chemsyn Chemical Services, Lenexa, Kansas) was used to investigate the fate of TNT incubated with the RLRW bacterial consortium. Heterotrophic medium (Section 3.2) was used with 100 mg/L of nonradioactive TNT. [^{14}C]TNT (19.3 µCi/mM in the experimental flasks) was added to the medium. The control flasks contained cells that were heat inactivated by autoclaving after inoculation. Samples were withdrawn periodically, and the quantity of TNT converted to biomass was determined as material precipitable by trichloroacetic acid (TCA) (Mans and Novelli 1961) by using a liquid scintillation spectrometer (Beckman Model LS 5000 TD). Samples of [^{14}C]TNT fractions were collected after passage through the high-performance liquid chromatography (HPLC) column, and the radioactivity in the fractions was measured by using a liquid scintillation spectrometer.

Respirometer flasks (Bartha and Pramer 1965) containing [^{14}C]TNT were used to monitor the CO_2 evolved by the bacterial consortium, with KOH (0.1 N) added to the side arms. The flasks were incubated at ambient temperature in a gyratory shaker at 90 rpm. The respirometers were sampled every two days by withdrawing KOH, measuring the radioactivity with a liquid scintillation spectrometer, and replacing the KOH. The percentage of [^{14}C]TNT mineralized as $^{14}CO_2$ was calculated. The experiments were conducted in duplicate and repeated.

3.10   Analysis of Cell-Bound TNT

To determine whether TNT was adsorbed to the cell and cell wall, cells were cultured in a large volume (1 L) in heterotrophic medium (Section 3.2). The culture was centrifuged. The

supernatant was discarded, and the cells were resuspended in methylene chloride. This procedure was repeated, and the supernatant was analyzed for TNT and TNT intermediates by using HPLC. This experiment was carried out with nonradioactive TNT.

In another experiment using radiolabeled TNT, the bacterial consortium was incubated in heterotrophic medium (Section 3.2) with nonradioactive TNT (100 mg/L) and [^{14}C]TNT (20,000 cpm). After all of the TNT was transformed, the culture was centrifuged, and the cells were harvested. The cells were resuspended in a phosphate buffer and broken open by using a french press. Radiolabeled TNT was measured in the disrupted cell materials and the supernatant by using a liquid scintillation counter.

3.11 Induction of TNT-Degrading Genes by Purines and Pyrimidines

The induction of TNT-degrading gene(s) in the RLRW bacterial consortium was evaluated by using purines and pyrimidines. (Pyrimidines are cytosine, thymine, and uracil, while the purines are adenine and guanine.) Bacterial cultures were pregrown in heterotrophic medium (Section 3.2) with 0.2% molasses and 0.05% each of purine or pyrimidine bases, which might induce the metabolism of TNT in the pregrown cells. The pregrown cells were inoculated into a mineral salt medium with TNT (100 mg/L) as the sole source of carbon and energy. Appropriate controls (one set without cells and another set containing cells not pregrown on inducing materials like purine and pyrimidine) were run. Growth and TNT concentration were monitored. The experiment was conducted in duplicate at room temperature, with cultures in a shaker set at 150 rpm.

3.12 Metabolism of TNT by Resting Cells and Cell-Free Extract

3.12.1 Resting Cells

Large volumes of cells were grown (1 L) in the heterotrophic medium described in Section 3.2, with succinate (5 g/L) as the carbon source. The culture was centrifuged at 20,000 rpm for 10 min. The supernatants were discarded, and the cells were resuspended in 10 mL of phosphate buffer (1 N K_2HPO_4 and 1 N KH_2PO_4 at pH 7). TNT (25 mg/L) was added to the resuspended cells. The cells were incubated at room temperature in a shaker set at 150 rpm. The TNT concentration was measured by using HPLC.

3.12.2   Cell-Free Extract

Large volumes of cells were grown (1 L) in the heterotrophic medium described in Section 3.2, with succinate (5 g/L) as the carbon source. The culture was centrifuged at 20,000 rpm for 10 min. The supernatants were discarded, and the cells were resuspended in 10 mL of phosphate buffer (1 N K_2HPO_4 and 1 N KH_2PO_4 at pH 7). The suspended cells were ruptured by passing them through a french press three times. Then the material was centrifuged at 20,000 rpm for 5 min. The supernatant was removed and transferred to a shake flask. TNT (25 mg/L) was added to the supernatant, and the mixture was incubated at room temperature in a shaker set at 150 rpm. The TNT concentration was monitored in the cell-free extract.

3.13   Studies on Pure Cultures

The RLRW bacterial consortium was plated repeatedly on TSA (Tryptic Soy Agar) plates (Becton Dickinson Microbiology Systems, Cockeysville, Maryland). After five transfers, colonies were restreaked and transferred to liquid cultures. Isolated colonies were characterized for their general morphology and microbial properties. Pure cultures were identified by gram staining and by the API rapid NFT Test (Analytab Products, Plainview, New York). The rates of TNT metabolism and growth were compared among the isolates. The heterotrophic medium described in Section 3.2 was used with succinate (5 g/L) as the carbon source. Duplicate culture flasks were maintained, and bacteria in the control flasks were heat inactivated by autoclaving after inoculation.

3.14   Soil Slurry Reactor

With the information gained from the previous experiments, we designed two 0.5-L aerobic/anoxic soil slurry reactors. The reactor setup is shown in Figure 1. TNT-contaminated soil was collected at the Joliet Army Ammunition Plant, Joliet, Illinois. The TNT concentration in the soil ranged from 10,000 to 20,000 mg/kg (Table 1). The laboratory-scale reactors were operated semicontinuously. The reactors were started with 15% (W/V) of TNT-contaminated soil. Molasses (0.3%) (Grandma's Molasses, Mott's USA, Cadbury Beverages, Inc., Stamford, Connecticut) served as the carbon source. Nitrogen in the form of NH_4Cl (0.1 g/L) (Fisher Scientific, Fair Lawn, New Jersey) was supplemented. Air was provided through a diffuser for 15-30 min each day. The soil slurry was mixed continuously at the rate of 90 rpm by using a magnetic stirrer. After a two-week stabilization period, 10% (W/V) of the contaminated soil was replaced every week, and carbon and nitrogen were added at the concentrations mentioned above. After 200 days of operation, the soil replacement was increased to twice weekly (10% soil replacement each time) in one reactor and three times weekly (10% soil each time) in another
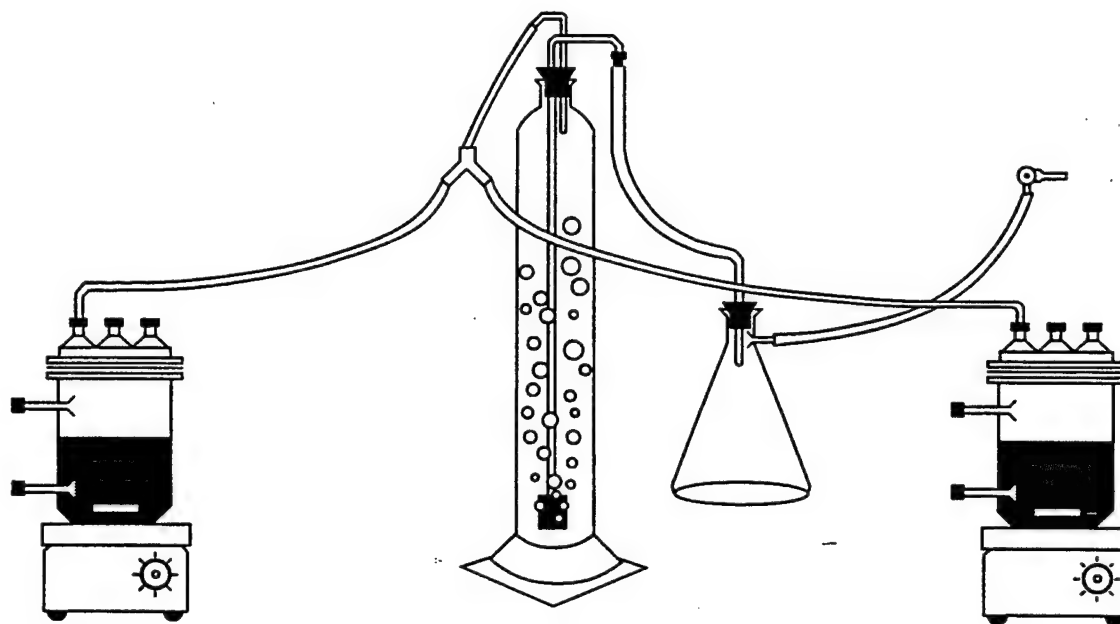
FIGURE 1 Schematic Diagram of the Soil Slurry Reactor

TABLE 1 Explosives Concentrations in Soil

Explosive[a]	Concentration Range (mg/kg)
TNT	10,000-20,000
Trinitrobenzene	175-300
2,4-Dinitrotoluene	50-200
RDX	50-125
HMX	50-100
Trinitrobenzaldehyde	50-150

[a] Abbreviations: HMX, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine; RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine; TNT, 2,4,6-trinitrotoluene.

reactor. Concentrations of TNT, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX), trinitrobenzene (TNB), 2,4-dinitrotoluene (DNT), nitrite, and ammonia; bacterial growth; pH; and oxygen uptake rates were monitored periodically.

3.15   Carbon-14 Mineralization Studies in the Slurry Reactor

After seven months of reactor operation, 100 mL of soil slurry was taken from each reactor. The soil slurry was incubated with [^{14}C]TNT (uniformly ring labeled) to establish mass balance and determine the production of metabolites including $^{14}CO_2$. The [^{14}C]TNT was added to the soil slurry in respirometer flasks at the level of 20,000 cpm/mL (Bartha and Pramer 1965). The control flask contained autoclaved soil slurry. Samples were withdrawn periodically, and the quantity of TNT converted to biomass was determined as TCA-precipitable material (Mans and Novelli 1961) by using a liquid scintillation spectrometer (Beckman Model LS 5000 TD). The CO_2 evolved from degradation of [^{14}C]TNT by the soil bacteria was monitored according to the method described by Bartha and Pramer (1965). KOH (0.5 N) was added to the side arms of the respiratory flasks. The flasks were incubated at ambient temperature in a shaker set at 50 rpm. The respirometer was sampled periodically by withdrawing KOH, measuring the radioactivity with a liquid scintillation spectrometer, and replacing the KOH. The percentage of [^{14}C]TNT mineralized as $^{14}CO_2$ was calculated. The experiment was conducted in duplicate.

The TNT metabolites were analyzed by collecting fractions every 30 s after passage through the HPLC column. The radioactivity in each fraction was measured by using a liquid scintillation counter. Soil-bound radioactive TNT was analyzed by using the soil extraction procedure described above, and the radioactivity in the soil was measured by using a liquid scintillation counter.

3.16   Identification of the Unknown Intermediate Generated in the Reactor Radiolabeling Study Described in Section 3.15

The intermediate that eluted at 2.2 min during the HPLC analysis was collected (by passage through the HPLC column), concentrated, and resuspended in acetonitrile. The concentrated sample was analyzed by gas chromatography/mass spectrometry (GC/MS) in the electron ionization mode on a Hewlett Packard model 5970 system. The samples were chromatographed with a gradient temperature program. The initial temperature of 100°C was held for 2 min, and then the temperature was increased to 280°C at 10°C/min and held at 280°C for 20 min. An SPB-5 (Supelco) (30 m × 0.25 mm, 0.25-μm film) column was used. The injection temperature was 280°C, and the transport line was kept at 220°C. The helium flow was 10 cm/s, and injection volumes were 1 μL.

4   Analytical Methods

4.1   TNT Analysis in the Aqueous Phase

The TNT was analyzed by HPLC with a Waters Associates (Milford, Massachusetts) liquid chromatograph equipped with two model 6000A solvent pumps, a model 990 variable photodiode-array multiple-wavelength detector set at 254 nm, a data module, and a model 600E system controller. The mobile phase was acetonitrile:methanol:water (21:35:44, V/V). Aliquots of 50 μL were injected into a Waters LC-18 μ-Bondopak column at room temperature. The flow rate was 1.5 mL/min. Samples were centrifuged at 5,000 rpm for 10 min, and the supernatant was filtered through 0.45-μm filters (Millipore, Bradford, Massachusetts). The filtrate was used for the analysis.

4.2   Analysis of Soil-Bound TNT

Soil-bound TNT was analyzed by using a soil extraction procedure developed by Jenkins and Walsh (1987). Soil slurry was oven dried, and 1 g of soil was extracted with 10 mL of acetonitrile. The whole mixture was sonicated for 18 h by using a sonicator (Solid State Ultrasonic Cleaning System, model FS 7652, Fisher Scientific, Itasca, Illinois). After sonication, 5 mL of the soil slurry extract was mixed with 5 mL of calcium chloride (5 g/L). Then the whole mixture was filtered through a 0.45-μm filter (Millipore). TNT and other contaminants in the filtrate were analyzed by HPLC with a Waters liquid chromatograph equipped with two model 6000A solvent pumps, a model 490E programmable multiwavelength detector set at 254 nm, a data module, and a model 600E system controller. The mobile phase was methanol:water (50:50 V/V). Aliquots of 50 μL were injected into a C-18 Supelco (Supelco, Inc., Bellefonte, Pennsylvania) column at 4°C. The flow rate of the solvent was 1.5 mL/min.

4.3   Analysis of Nitrite

The nitrite concentration in aqueous solution was determined by a colorimetric method through the formation of a reddish-purple azo dye produced at pH 2.0-2.5 by coupling diazotized sulfanilic acid with N-(1-naphthyl)-ethylenediamine dihydrochloride (APHA 1988). This method is suitable for determination of nitrogen as nitrite down to 1 μg/L. Reagent grade sodium nitrite (Fisher Scientific, Fair Lawn, New Jersey) was used as the standard for calculating the nitrite concentrations in the samples.

4.4   Analysis of Ammonia

The ammonia concentration in soil slurry samples was analyzed by a colorimetric method using a Hach water analysis reagent kit (Hach Company, Loveland, Colorado). The 5-mL slurry sample was prepared for analysis by centrifuging (Dynac II centrifuge, Becton Dickinson) it for 10 min at 4,000 rpm. The supernatant was filtered through a 0.45-µm filter (Millipore). The filtrate used for the ammonia analysis was diluted 25 times in water. Three drops each of mineral stabilizer and polyvinyl alcohol were added to the diluted sample. Then 1 mL of Nessler reagent was added. After vigorous mixing, the yellow color developed was read at 425 nm by using a Spectronic 20 spectrophotometer. The mineral stabilizer complexes the calcium and magnesium salts in the sample. The polyvinyl alcohol dispersing agent aids color formation in reactions of Nessler reagent with ammonium ions. Reagent-grade ammonium chloride (Fisher) was used as a standard for calculating the concentrations of ammonia in the samples.

4.5   Oxygen Uptake Rates

Oxygen uptake rates in the soil slurry were monitored by using an oxygen analyzer (Gilson Oxygraph, model Oxy-5, Gilson Electronics, Inc., Middleton, Wisconsin). A sample containing bacterial cells was added to the cell, and the initial oxygen level (the baseline) was recorded on a plotter. After the baseline was established, the nutrient solution containing carbon and nitrogen was added to the bacteria in the cell, and oxygen uptake was monitored for 5 min. The difference between the baseline and the level at the end of the analysis was taken as the oxygen uptake rate, expressed as mg/L.

4.6   Analysis of Intermediates

The TNT intermediates were identified by using GC/MS. Peaks of interest on liquid chromatograms were collected, concentrated, resuspended in acetonitrile, and analyzed by GC/MS. The GC/MS analyses were performed in the electron ionization mode on a Finnigan model INCOS 50 system. The samples were chromatographed on a temperature gradient. The initial temperature of 80°C was held for 1 min, after which the temperature was increased to 250°C at 20°C/min and then held at 250°C for 10 min. The column used was SPB-5 (Supelco) (30 mm × 0.25 mm, 0.25-µm film). The injector temperature was 250°C, and the transport line was kept at 200°C. The helium flow rate was 15 mL/s, and injection volumes were 2 µL.

4.7   Growth

Culture turbidity was measured by absorbance at 600 nm with a Spectronic 20 spectrophotometer. Growth in the slurry reactor was monitored by total plate counts on TSA plates.

4.8   Chemicals

Radiolabeled TNT (uniformly ring labeled; specific activity 21.58 mCi/mM, 98.5% pure) was purchased from Chemsyn Science Laboratories, Lenexa, Kansas. The nonradioactive TNT (98% pure) was obtained from Chem Service, Inc., Westchester, Pennsylvania. The TNB, DNT, RDX, and HMX were obtained from the Naval Surface Warfare Center, Indian Head, Maryland, through the USAEC's Standard Analytical Reference Material Program. All other chemicals were of reagent grade.

5   Results and Discussion

5.1   Development and Screening of the Bacterial Consortium

Soil samples of TNT-contaminated soil were collected at the Joliet Army Ammunition Plant, Joliet, Illinois. The bacterial consortium from the soil samples was cultured in Stanier's medium (Stanier et al. 1966) with and without succinate. No growth was observed in the cultures without succinate as a co-substrate, but the cultures receiving succinate as co-substrate at 0.5% (W/V) did show better growth (Figure 2). Of the four different consortia obtained, one consortium, named RLRW, was selected for further experiments on the basis of its growth rate and TNT removal rate. The RLRW consortium was cultured on Stanier's medium and the heterotrophic medium described in Section 3.2. The consortium grew faster on the heterotrophic medium (Figure 3), so the heterotrophic medium was used in the rest of the study.

5.2   Biological Transformation of TNT

Figures 4 and 5 show the concentrations of TNT and nitrite and the growth of the RLRW bacterial consortium as a function of incubation time under aerobic conditions. In the uninoculated control, the TNT concentration did not decrease but remained at the original concentration of 100 mg/L. The flasks that contained TNT as the sole source of carbon and energy showed no growth, and the concentration of TNT remained at 100 mg/L. Bacterial growth was observed only in flasks containing succinate. The concentration of TNT in the succinate-containing flasks began to decrease within 24 h of incubation. After about 48 h, 78% of the TNT had disappeared from the culture medium. After 108 h of incubation, the TNT was 100% transformed. These results demonstrate that the bacterial consortium could not grow on TNT as a sole source of carbon and energy but could transform TNT by co-metabolism with succinate as the primary substrate. Maximal growth was observed at about 48 h of incubation, by which time 78% of the TNT was transformed. The nitrite concentration in the culture began to increase after 50 h of incubation. A maximum nitrite concentration of 620 µg/L was observed after 400 h of incubation. In the uninoculated control, the nitrite level was zero. No nitrite was produced in flasks containing no TNT.

The HPLC analysis of the culture supernatant showed that at least two intermediate products were present. Figure 6 shows the HPLC analysis of the 0-h- and 70-h-old cultures. The retention time of the TNT standard under the chromatographic conditions used in this study was 6.9 min. For the 0-h culture, the only peak eluted at 6.9 min, corresponding to the standard TNT. The chromatogram of the 70-h culture had a TNT peak and two other peaks that eluted before TNT. The TNT peak was significantly reduced, and the two intermediates originating from TNT metabolism eluted at 1.9 and 6.4 min. In the 70-h-old uninoculated control, only the TNT
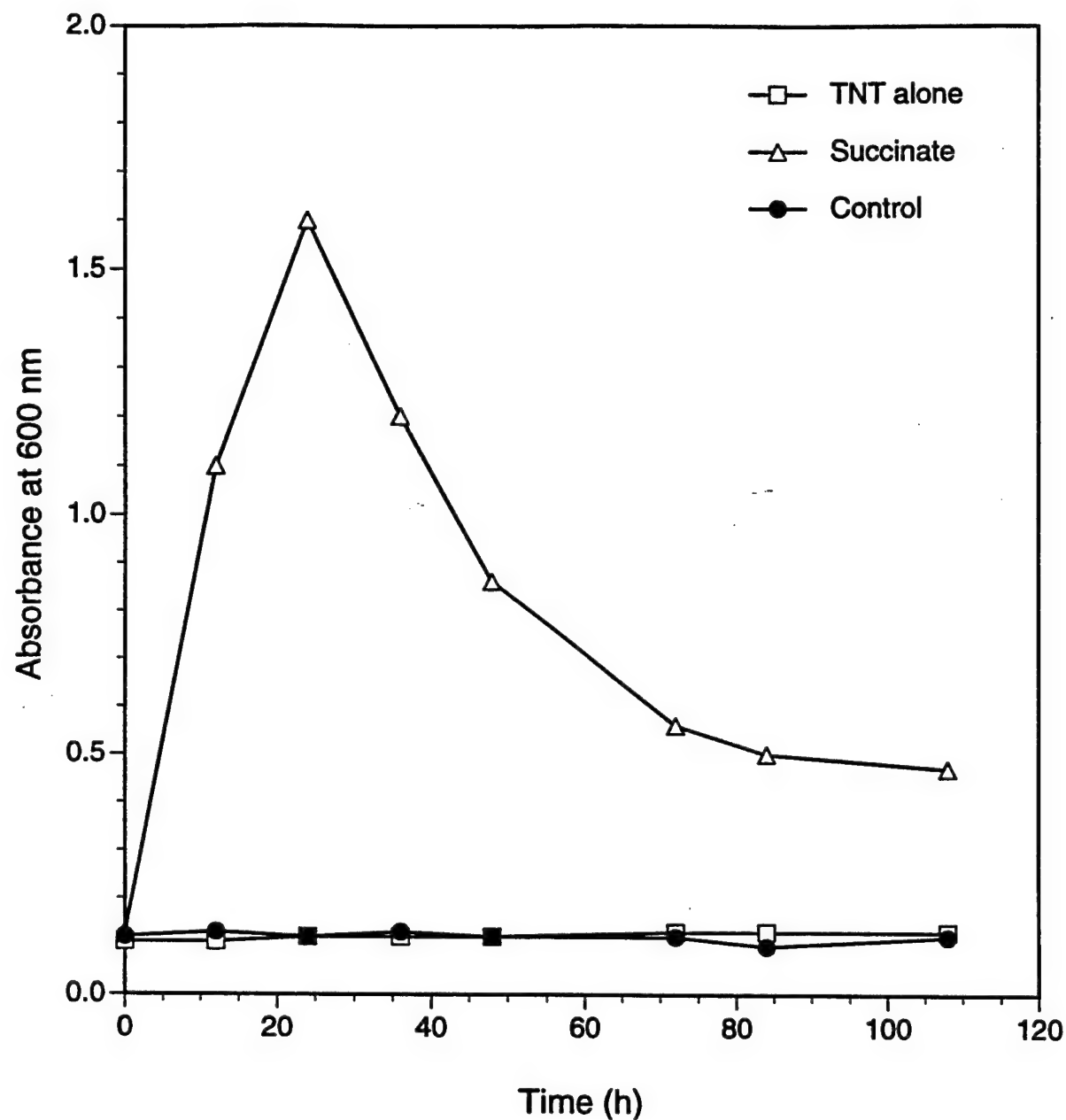
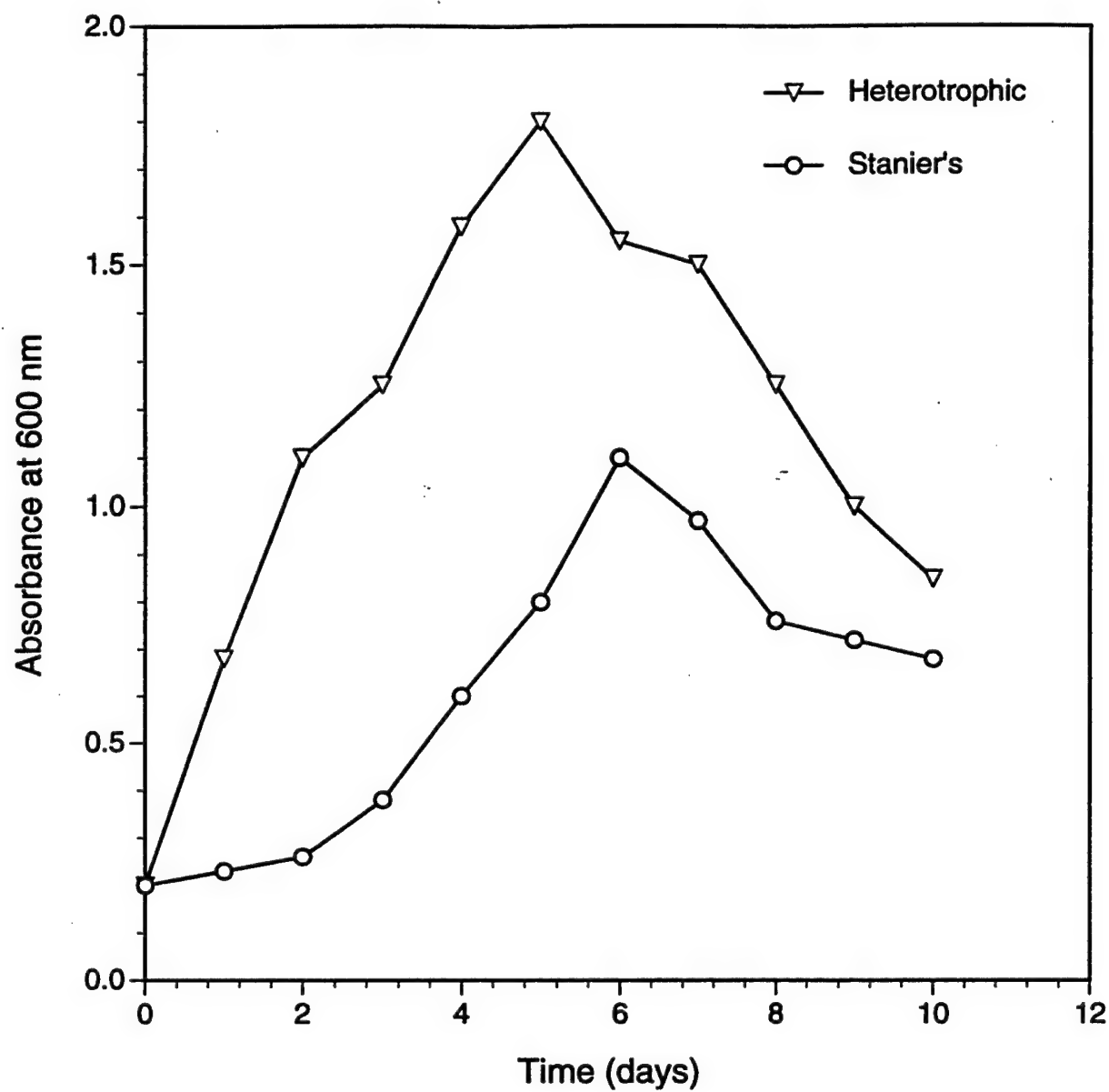FIGURE 2 Growth of RLRW with and without Succinate
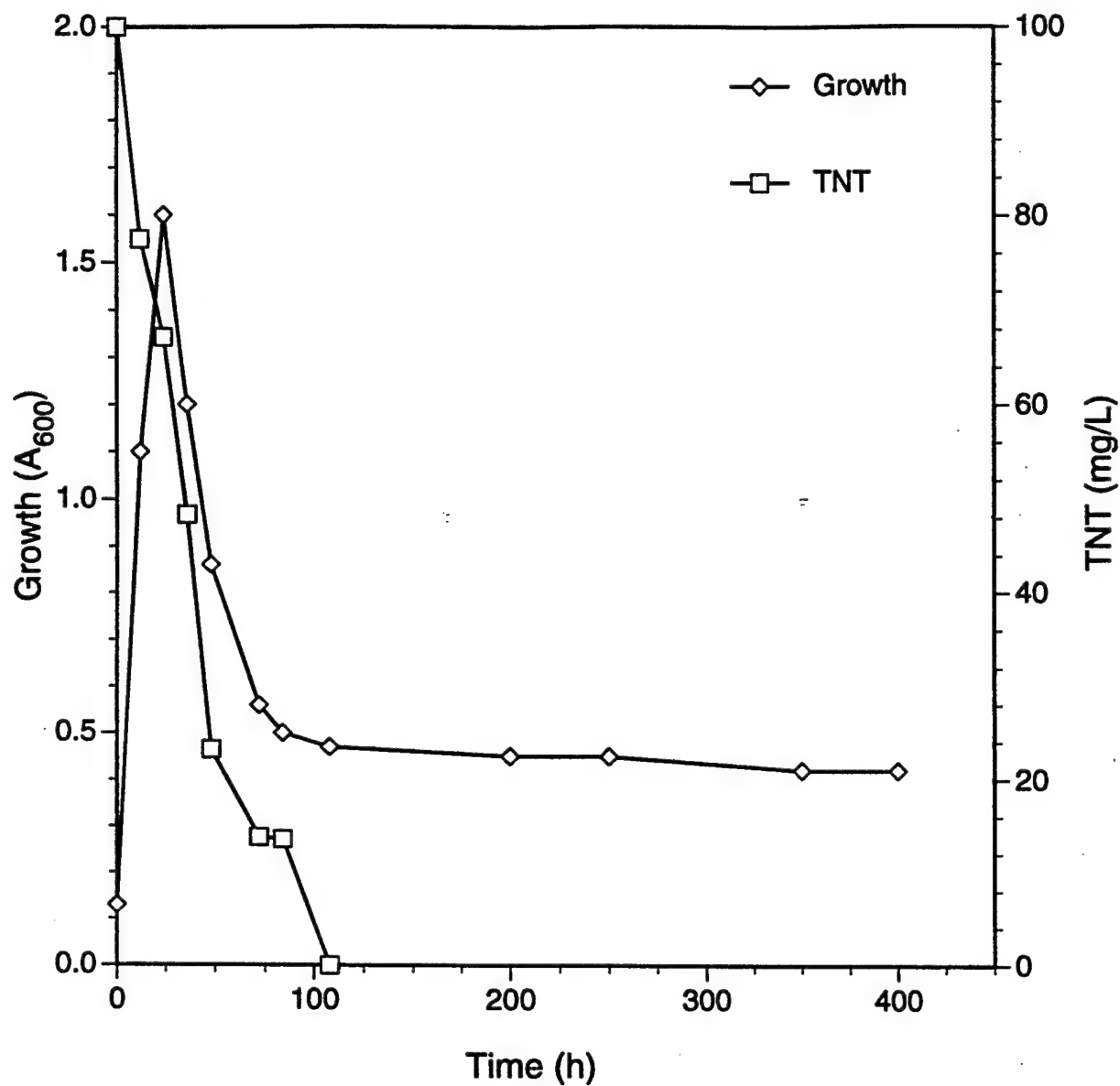
FIGURE 3 Growth of RLRW on Different Media

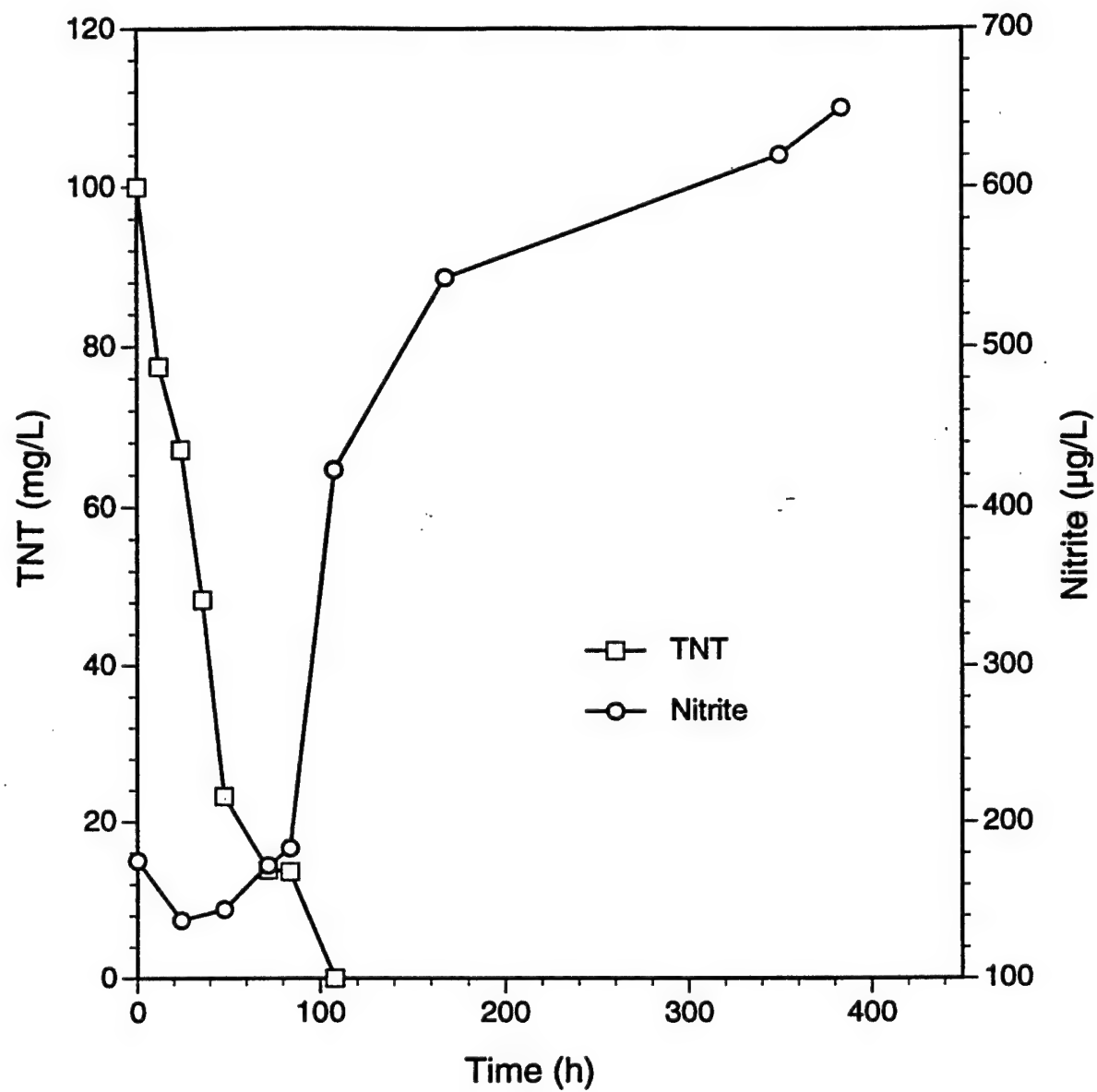FIGURE 4 Growth of RLRW and TNT Concentration

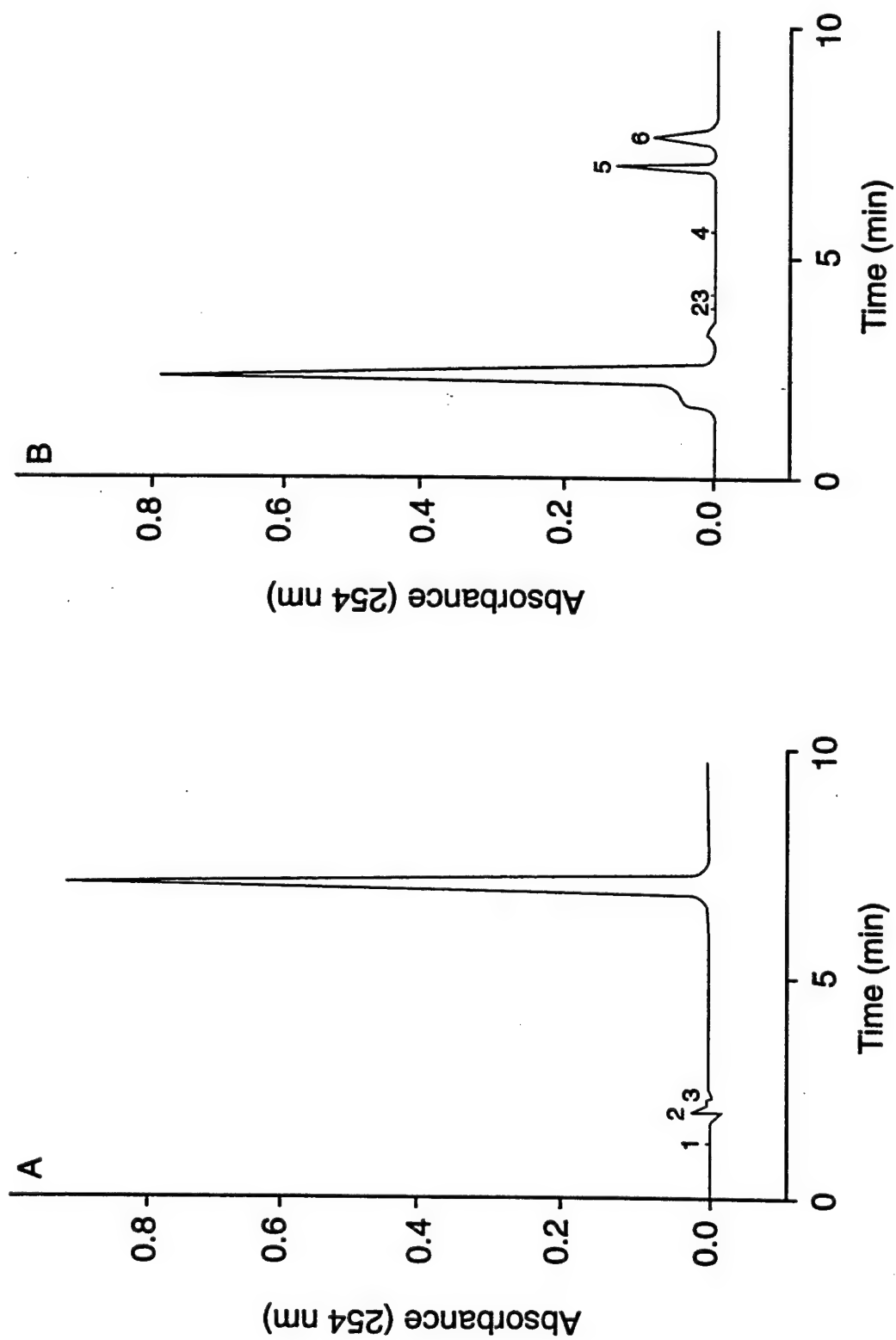FIGURE 5 TNT and Nitrite Concentrations in Aerobic RLRW Cultures

FIGURE 6   Elution Profile of TNT on Reversed-Phase HPLC (A, 0-h culture; B, 70-h culture)

peak eluted, at the same intensity as in the 0-h culture (chromatogram not shown). This absence of intermediate peaks indicates that the transformation of TNT is a biological process. The culture that received succinate but no TNT produced chromatograms devoid of peaks. This result showed that all of the intermediates originated from TNT. When no succinate or other carbon source was in the medium, no significant change in the concentration of TNT and no production of intermediates were observed. This finding confirms that the biotransformation of TNT in our system is a co-metabolic process.

5.2.1 TNT Metabolites

The GC/MS analysis of the culture sample showed two intermediates. The mass spectrum of the first intermediate gave a fragmentation profile similar to· that of authentic 4-amino-2,6-dinitrotoluene as shown by Pereira et al. (1979). The mass spectrum of the second intermediate closely resembles that of standard 2-amino-4,6-dinitrotoluene (Pereira et al. 1979) (Figure 7). Thus, the two major metabolites separated by HPLC analysis were identified as 4-amino-2,6-dinitrotoluene and its isomer, 2-amino-4,6-dinitrotoluene.

5.2.2 Radiolabeling Study

The protocol and analytical procedures for the radiolabeling experiment were discussed in Section 3.15. The aerobic bacterial consortium mineralized 3.1% of the $[^{14}C]$TNT to $^{14}CO_2$ after 70 days of incubation. The control showed $^{14}CO_2$ evolution of less than 0.7% (Figure 8). The amount of $[^{14}C]$TNT converted to cell mass by the bacterial consortium grown with succinate was determined by acid precipitation with TCA. Figure 9 shows that 8% of the $[^{14}C]$TNT was converted to biomass after 14 days of incubation. In the heat-killed control, less than 0.5% of $[^{14}C]$TNT was found in the biomass as TCA-precipitable material. This result indicates that TNT was used to make cell materials. The evolution of $^{14}CO_2$ and the production of nitrite indicate that the ring may have been cleaved.

This study showed that an aerobic soil bacterial consortium isolated from TNT-contaminated soil can cause extensive transformation of TNT in a short period of time. Slight degradation was demonstrated by mineralization of $[^{14}C]$TNT, metabolite formation, and the presence of $[^{14}C]$TNT in the cell biomass as TCA-precipitable material. The two major intermediates initially produced from TNT metabolism were reduction products identified as 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene.

In addition to the extensive production of intermediates, the bacteria were able to mineralize TNT to a limited extent. The radiolabeling experiments showed that prolonged incubation of the bacterial consortium, up to 70 days, converted $[^{14}C]$TNT into $^{14}CO_2$. Since the ring carbons of
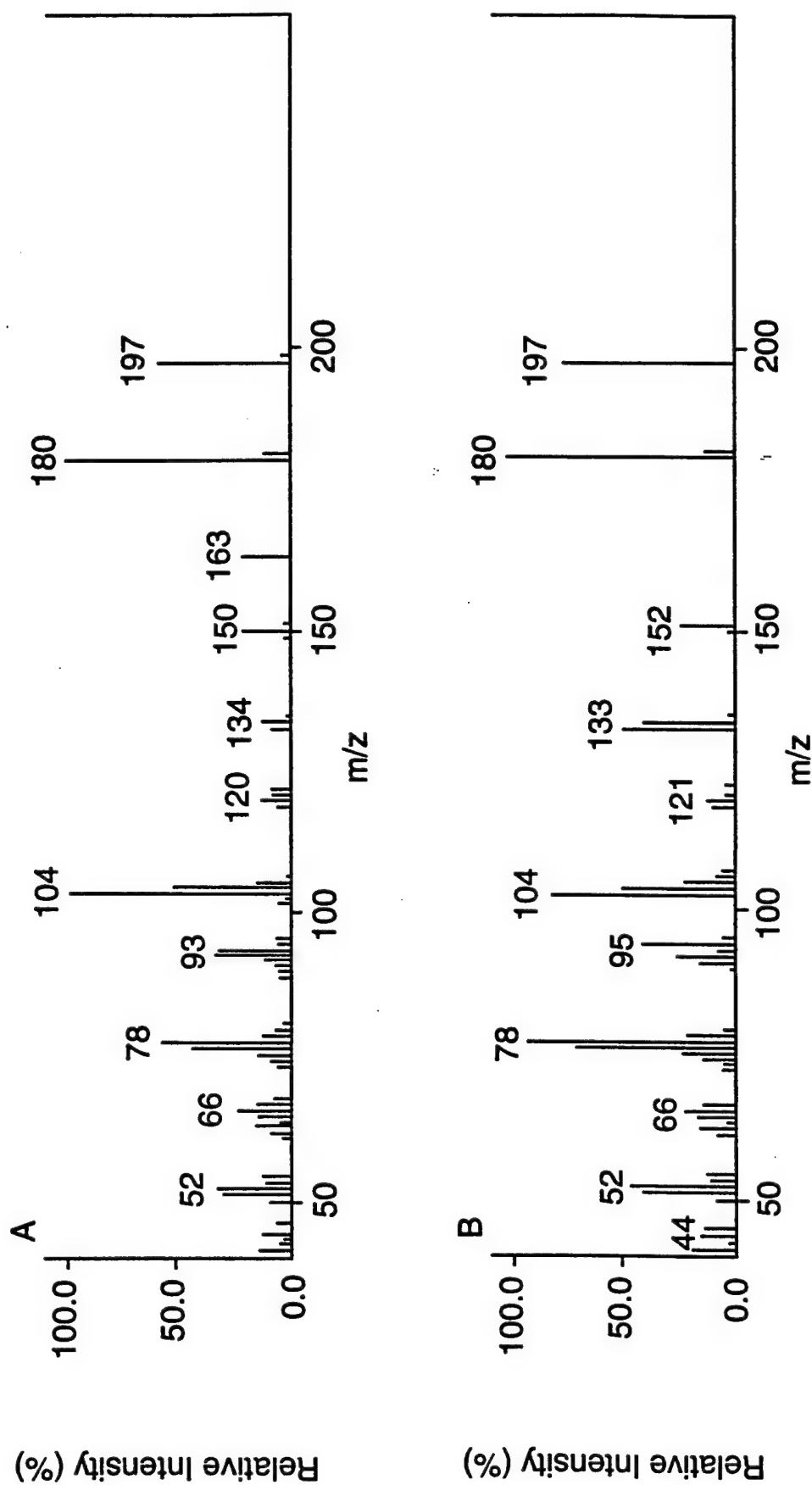
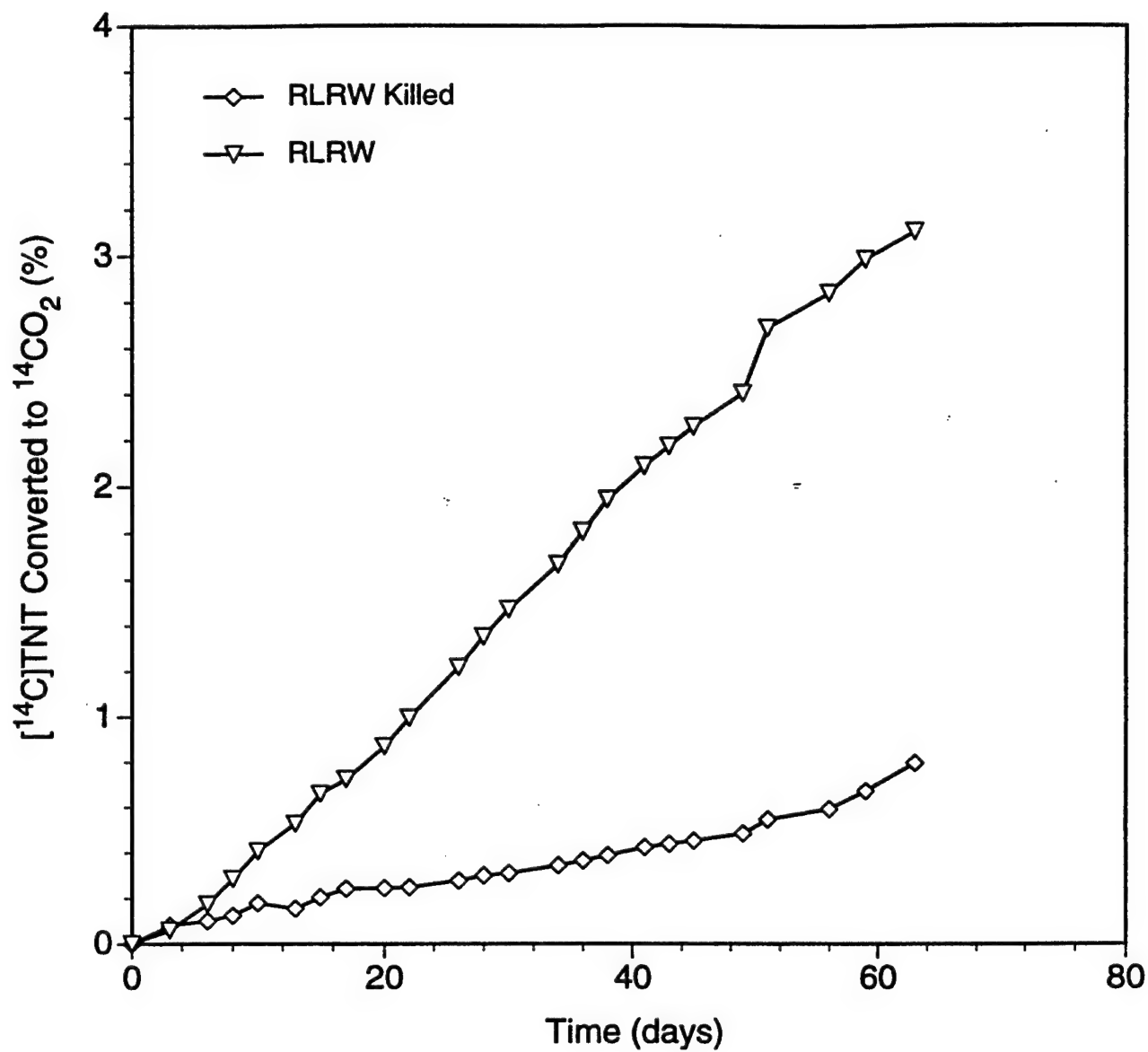FIGURE 7 Mass Spectra of the Metabolites (A, metabolite 1; B, metabolite 2)

FIGURE 8 Mineralization of $[^{14}C]$TNT to $^{14}CO_2$

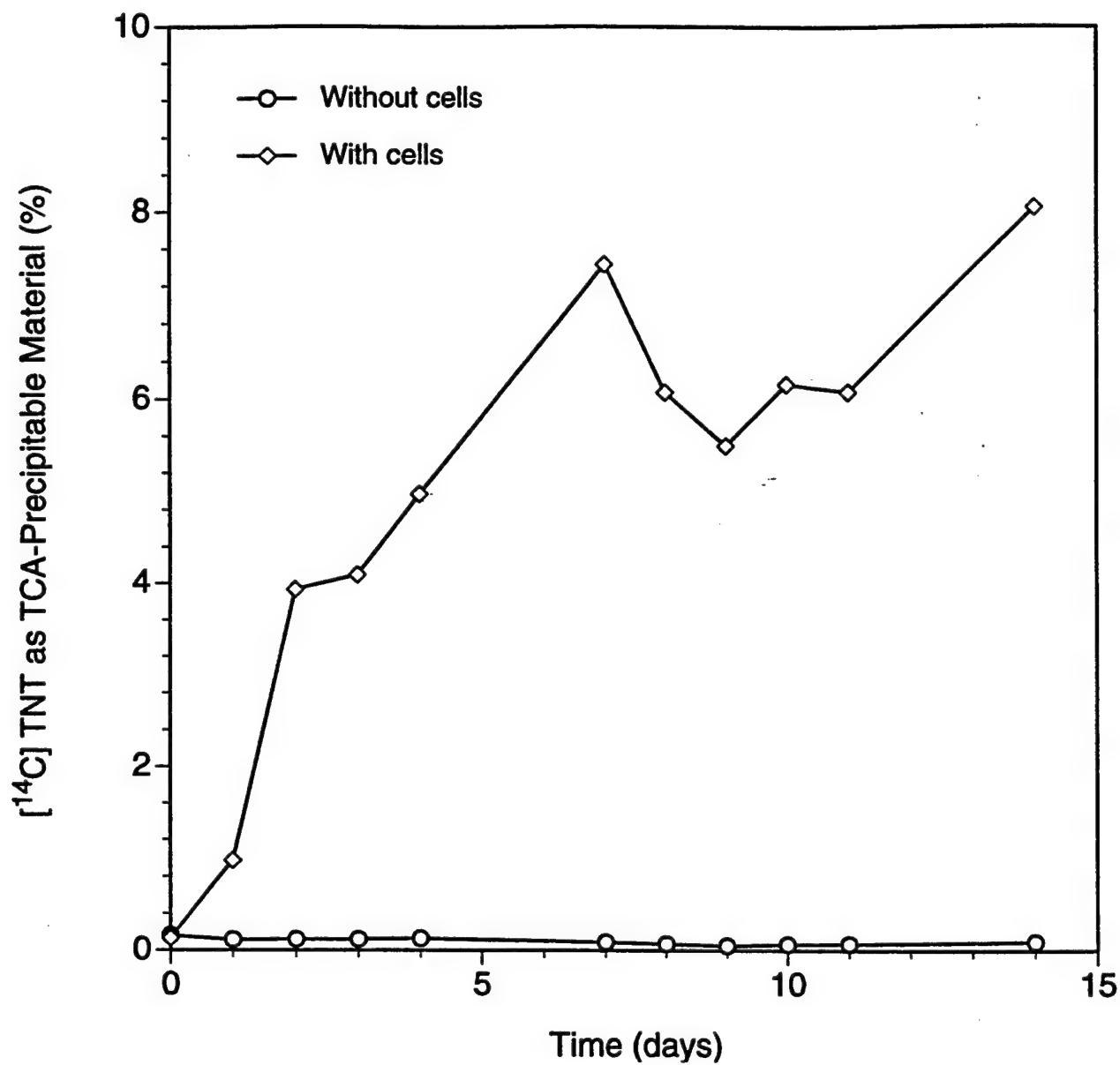FIGURE 9   TCA-Precipitable Material in Culture with RLRW Consortium

TNT were uniformly labeled, conversion to CO_2 clearly denotes ring cleavage. The rate of conversion was very slow, reflecting possible difficulty in metabolizing the intermediates of TNT to CO_2. Only 3.1% of [^{14}C]TNT was mineralized, and 8% of the TNT was converted to biomass. These data provide evidence that TNT was metabolized in a co-metabolic process. The mechanism of TNT ring cleavage is not clear.

The nitrite concentration in the culture medium increased after the bacterial consortium reached the stationary phase of growth, after all the TNT in the culture medium was transformed. The produced nitrite was equal to 11% of the nitrogen available from the nitro groups of the TNT.

5.3   Co-metabolic Screening Study

The performance of the RLRW bacterial consortium in transforming TNT in the presence of various substrates is presented in Table 2. The specific growth rate of the culture growing on molasses was three to six times those of cultures growing on succinate, citrate, malic acid, acetate, sucrose, or glucose. Similarly, the specific transformation rate of TNT in the molasses-fed culture was higher by an order of magnitude than those of cultures fed with other carbon sources. No growth or TNT transformation was observed in killed controls or in the cultures that received TNT as the sole source of carbon and energy. The molasses-fed culture took only 12 h to transform 100 mg/L of TNT, whereas the succinate-fed culture took 105 h, and the other cultures needed 130 h to transform 80-100 mg/L of TNT. The concentration of substrate used was significantly less for molasses (0.3%) than for the other substrates (0.5%). These results indicate that molasses is a very good substrate, stimulating bacterial growth and increasing the rate of TNT transformation. Molasses used in these experiments was commercial black strap molasses. The complex composition of molasses (with sugars, amino acids, and proteins) makes it conducive to the growth of many types of bacteria in the consortium.

Figure 10 shows the growth of the soil bacterial consortium with different concentrations of molasses. Molasses at 0.3% supported maximal growth. Increases in molasses concentration above 0.3% did not increase the growth rate (data not shown). The killed control and the cultures that received TNT as the sole source of carbon and energy showed no growth, even after 30 days of incubation. Figure 11 shows the TNT concentration in molasses-fed cultures. Molasses at 0.3% produced a higher TNT removal rate than molasses at 0.1% and 0.2%. Within 12 h of incubation, 100 mg/L of TNT was transformed with a molasses concentration of 0.3%. Further increases in molasses concentration did not significantly change the rate of TNT transformation (data not shown). No change occurred in the TNT concentration in control and non-molasses-fed cultures.

Radioactive labeling studies indicated that no significant production of $^{14}CO_2$ occurred. This result suggests that the TNT was not mineralized but was transformed. The HPLC analysis

TABLE 2 Transformation of TNT by Bacterial Cultures with Different Co-substrates

Substrate[a]	Specific Growth Rate (h - 1)[b]	TNT Concentration (mg/L)		Percent TNT Transformed	Specific Transformation Rate (mg/L·h)[c]
		Initial	Final		
Control	-	100	99	1	0.007
TNT alone	-	100	98	2	0.014
Malic acid	0.044	100	11.8	88.2	0.68
Acetate	0.034	100	14.08	85.92	0.66
Citrate	0.032	100	5.63	94.37	0.73
Sucrose	0.030	100	10.79	89.21	0.69
Glucose	0.034	100	7.41	92.57	0.71
Succinate	0.050	100	0	100	0.93
Molasses	0.18	100	0	100	8.3

[a] All substrates except molasses were at 0.5% concentration. Molasses concentration was 0.3%.

[b] Specific growth rates at the exponential phase of growth were calculated from plots of A_{600} versus time for the respective cultures.

[c] Specific transformation rates were calculated from the amount of TNT transformed and the time required for the transformation.
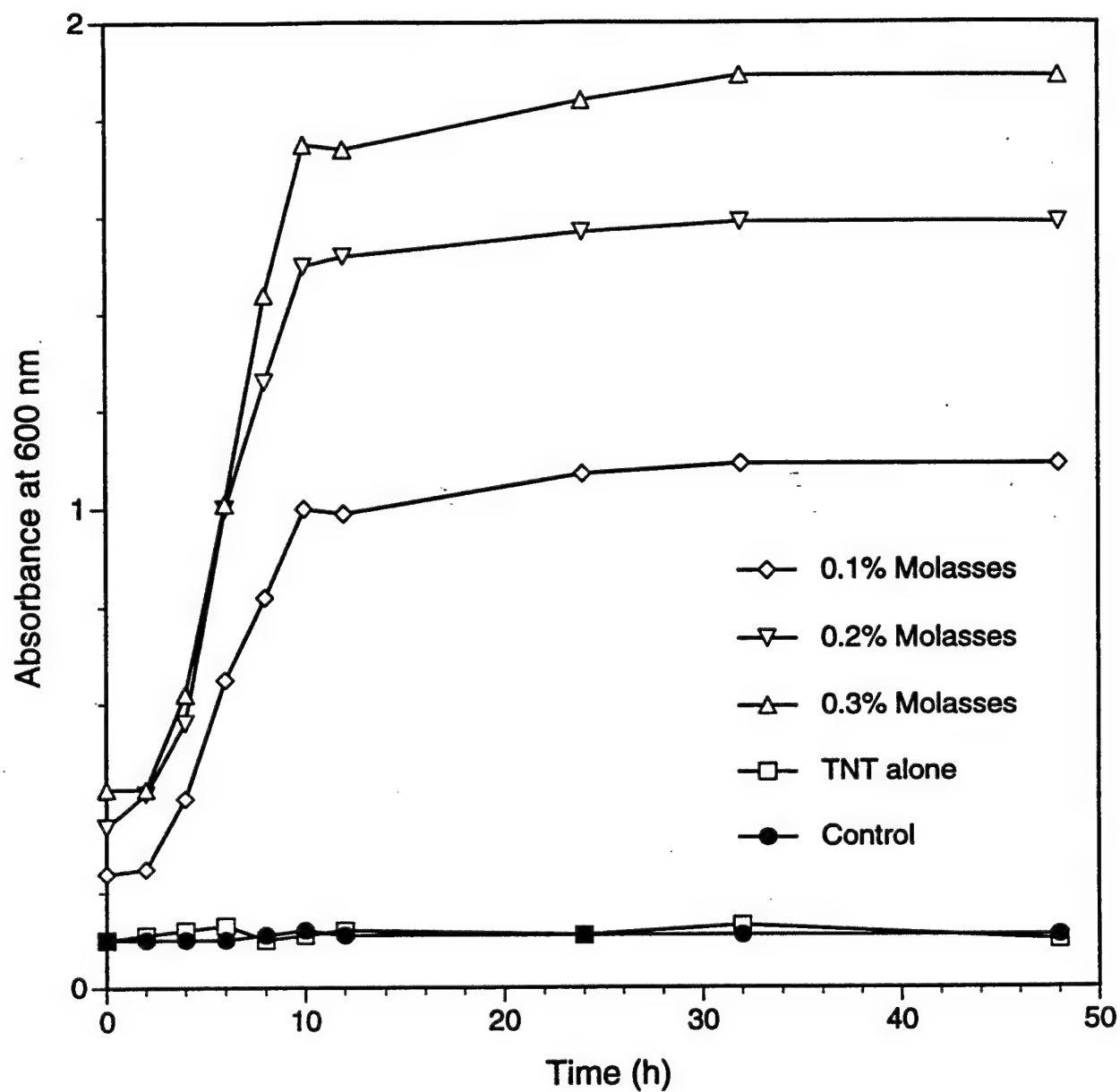
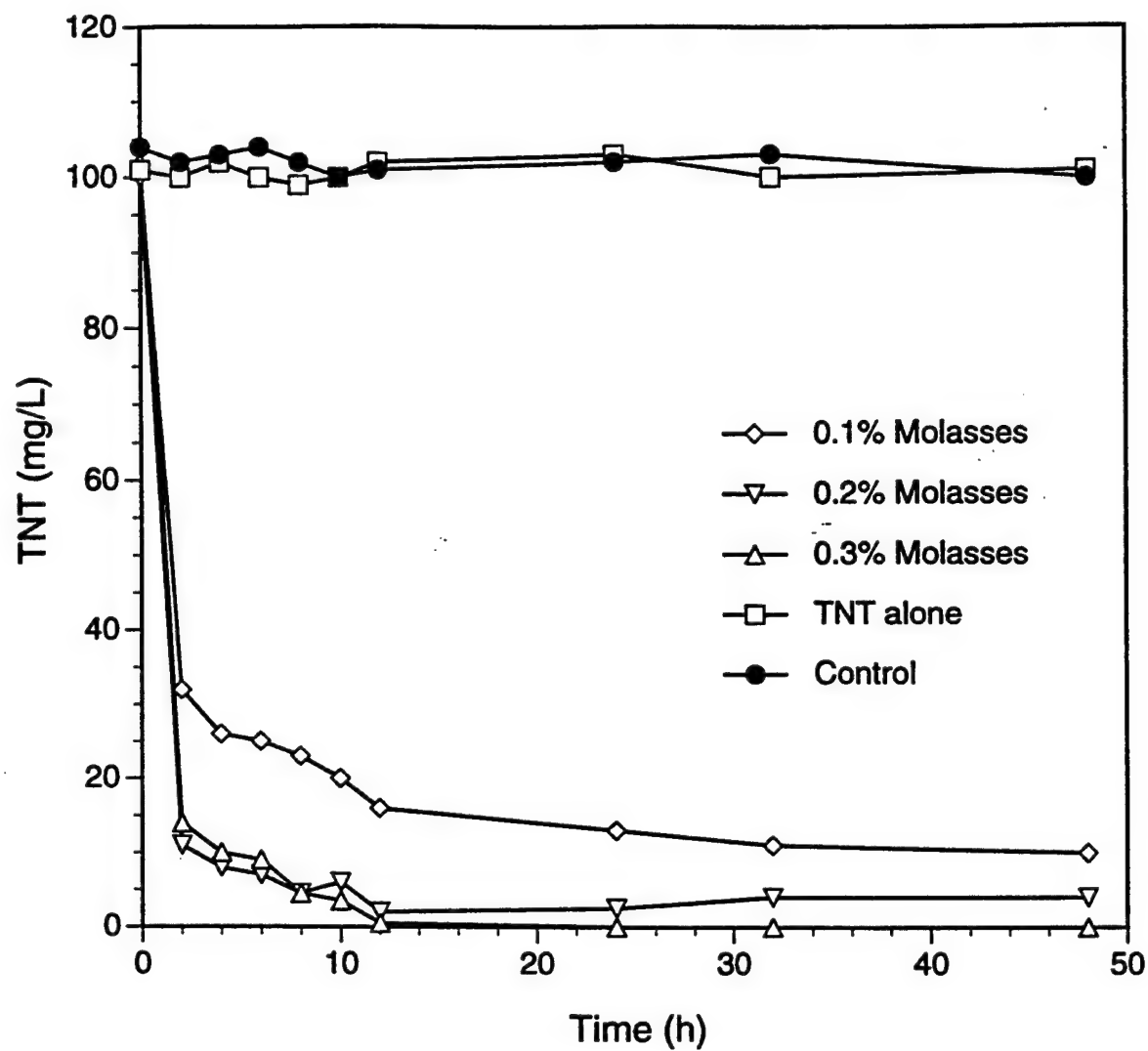FIGURE 10 Growth of the Bacterial Consortium on Molasses

FIGURE 11 TNT Concentration in Cultures Grown on Molasses

(Figure 12) of the [^{14}C]TNT demonstrates that 93% of the [^{14}C]TNT initially present could be identified as undegraded TNT metabolites. The GC/MS analysis of these intermediates identified them as 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene.

This work with molasses demonstrated that a variety of substrates can support co-metabolism of TNT. Among the different substrates studied, the relatively inexpensive molasses was found to produce the best results in terms of bacterial growth and TNT transformation. The cost of molasses is significantly less than the cost of other substrates, giving an added advantage that will make the large-scale TNT biotreatment system economically feasible.

5.4   Optimization Studies

To determine the optimal environmental parameters for bacterial growth, optimization studies in shake flasks were carried out at various temperatures, pH levels, and nitrogen concentrations as explained in Section 3.

5.4.1   Optimal Temperature

The effect of temperature on the growth of the RLRW bacterial consortium is shown in Figure 13 along with the percent of TNT degraded at various temperatures after 24 h of incubation. The growth was best at 25°C and 30°C. Ambient temperature, 22-24°C, also supported good growth. Growth at 37°C was lower than at other temperatures studied. Similarly, the percent degradation of TNT at various temperatures indicated that the cultures incubated at room temperature, 25°C, and 30°C performed better than the cultures incubated at the higher temperature of 37°C. This experiment showed that the soil bacterial consortium does not need the elevated temperature of 37°C.

5.4.2   Optimal pH

An experiment was conducted in shake flasks to determine the optimal pH for growth of the soil bacterial consortium. The growth of RLRW and the percent TNT degradation are shown in Figure 14. As expected, growth was better at pH 7 than at pH 6, 6.5, 7.5, or 8. On the other hand, a slight difference was observed in the percent of TNT degraded at the various pH levels studied. After a 24-h incubation period, 93% of the TNT was degraded at all of the pH levels. This experiment demonstrated that even though pH 7 supported maximum bacterial growth, a range of pH levels between 6 and 8 will support TNT degradation.
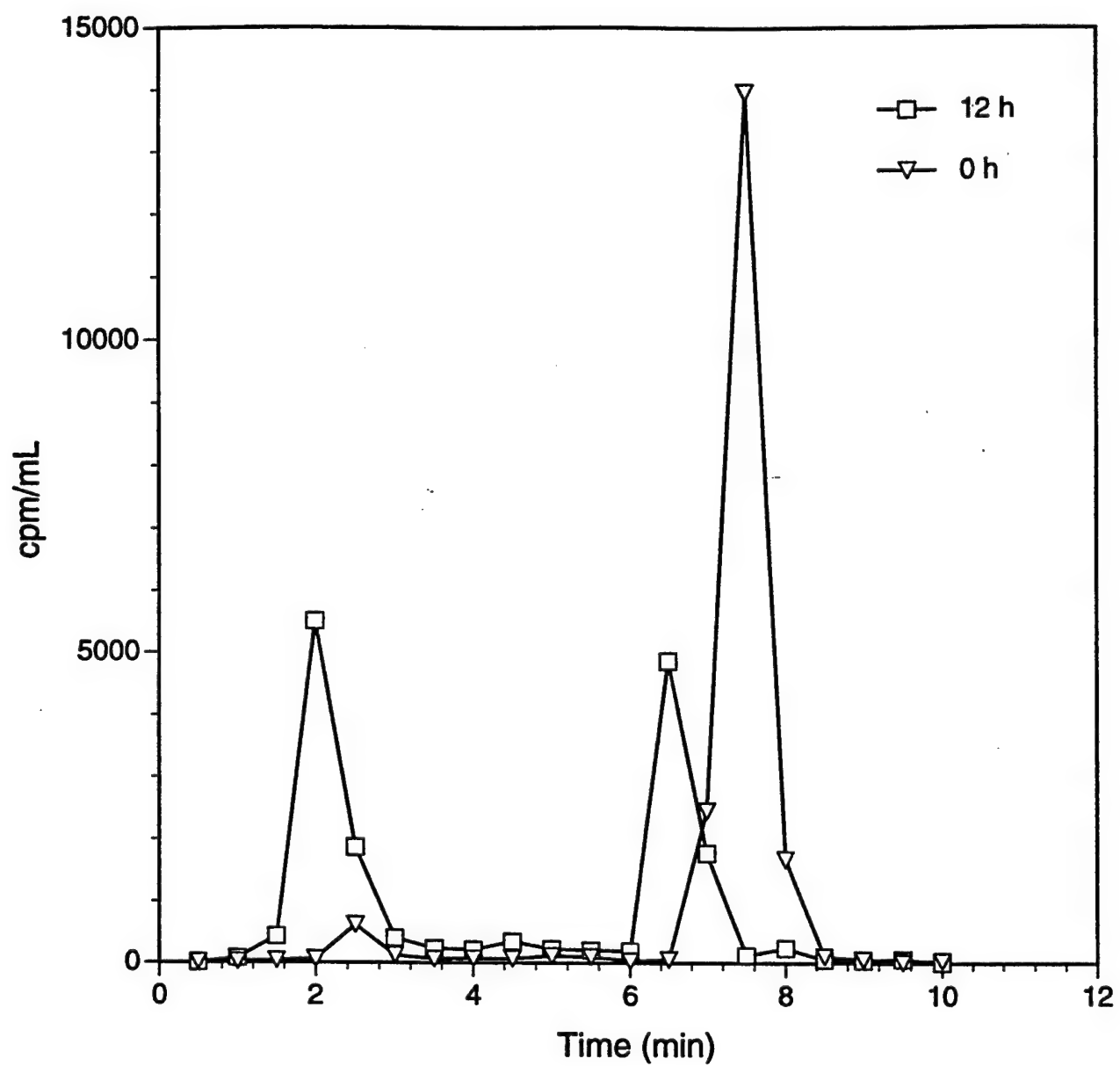
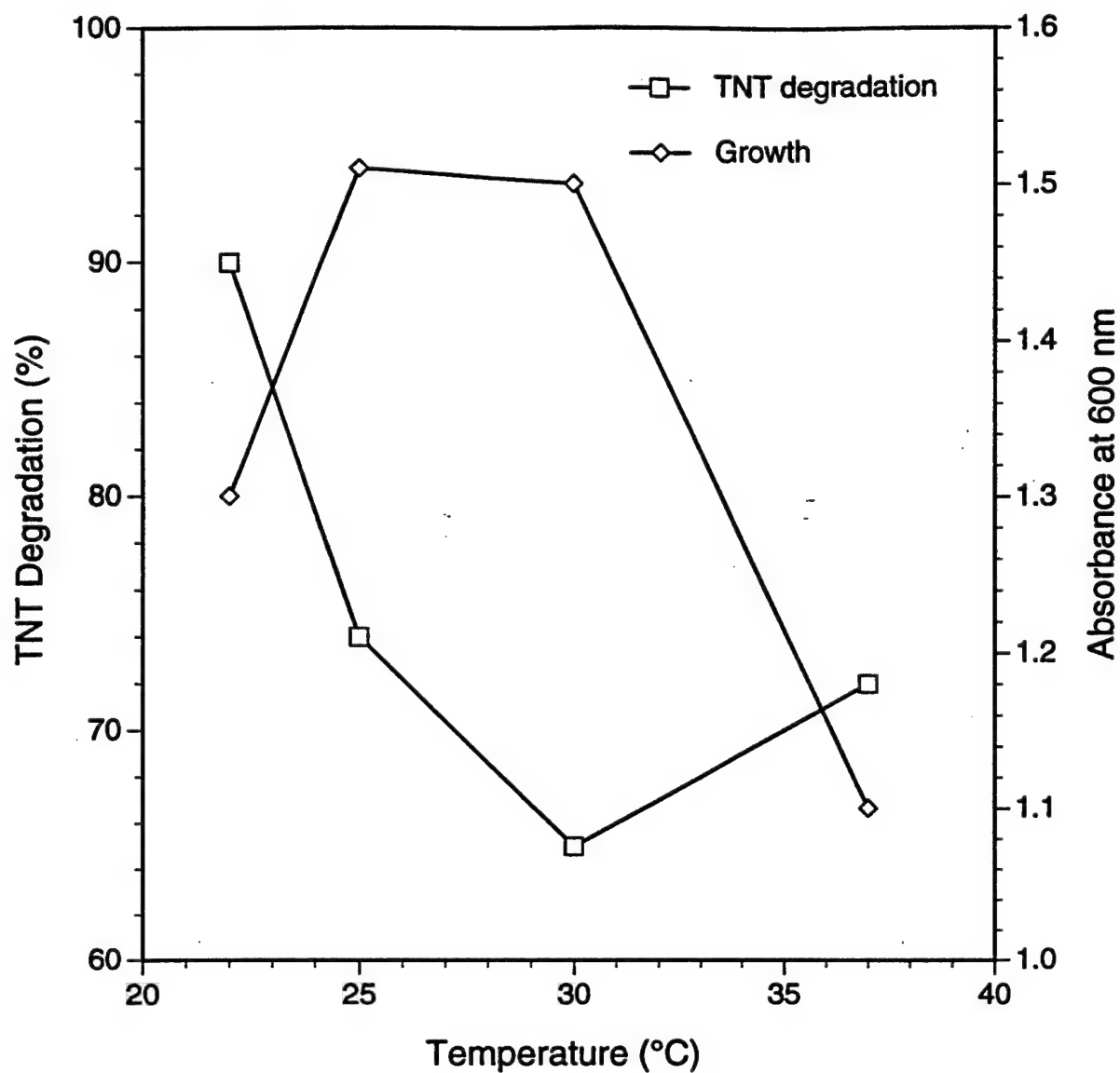FIGURE 12 HPLC Elution Profiles of 0-h and 12-h Cultures

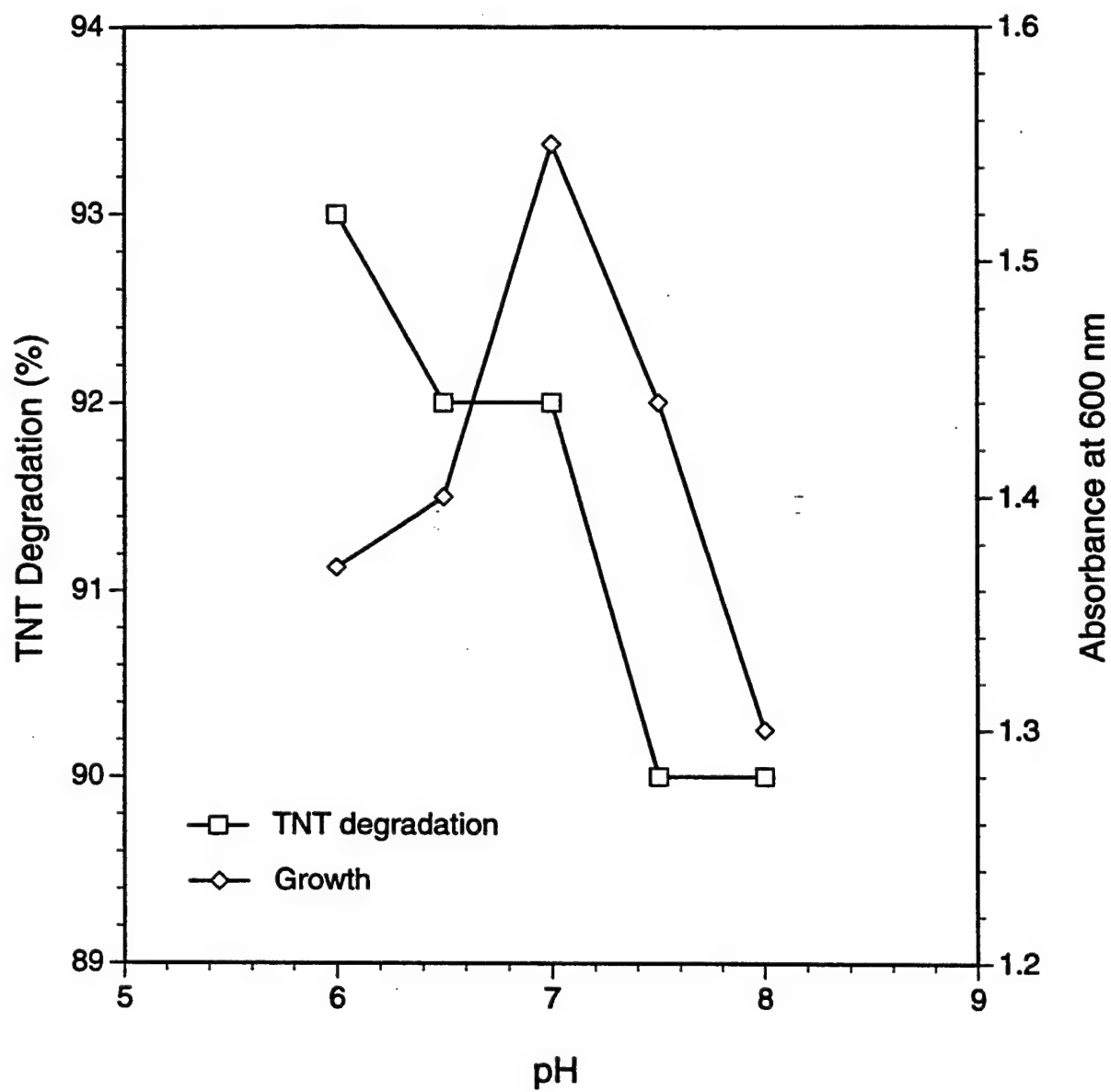FIGURE 13 Effect of Temperature on TNT Metabolism

FIGURE 14 Effect of pH on TNT Metabolism

5.4.3 Nitrogen Requirement

In an experiment to see whether the RLRW bacterial consortium can use TNT as its nitrogen source, the heterotrophic medium was used. One set of cultures received TNT alone as the nitrogen source, and another set received TNT plus ammonium chloride (0.5 g/L) as an inorganic nitrogen source. Succinate (5 g/L) served as the carbon source. Figure 15 shows the TNT concentration in the culture medium under the nitrogen-limiting and nitrogen-rich conditions. The TNT concentration decreased slightly (10%) in the cultures that received TNT alone as the supplemental nitrogen source, whereas in the cultures that also received ammonium chloride as an inorganic nitrogen source the TNT concentration was reduced by more than 90% (Figure 15). This experiment showed that the soil bacterial consortium could not use TNT as its sole source of nitrogen and that additional nitrogen in the form of ammonium chloride had to be supplied for the metabolism of TNT.

In another experiment an attempt was made to determine the level of ammonium chloride that must be supplied. The RLRW bacterial consortium was incubated in the heterotrophic medium with various concentrations of ammonium chloride. The cultures that received TNT as a nitrogen source did not grow. Ammonium chloride concentrations of 0.5 and 1.0 g/L supported bacterial growth equally well (Figure 16). This study showed that for good bacterial growth ammonium chloride should be supplemented at a level of 0.5 g/L.

5.5 TNT Metabolism under Nitrate-Reducing Conditions

In an experiment conducted to study the metabolism of TNT by the aerobic RLRW bacterial consortium under nitrate-reducing conditions, cultures were incubated under anoxic conditions in serum bottles. Sodium nitrate (20 mM) served as the electron acceptor. The details of the experiment are discussed in Section 3.7.

Figure 17 illustrates the bacterial growth under aerobic conditions and nitrate-reducing conditions. The results indicate that the bacterial consortium is a strict aerobe and that it did not grow under anoxic, nitrate-reducing conditions. Similarly, no metabolism of TNT occurred under nitrate-reducing conditions. On the other hand, under aerobic conditions 100% of the TNT was transformed within 24 h of incubation (Figure 18). This experiment showed that the bacterial consortium obtained from the TNT-contaminated soil metabolizes TNT only under aerobic conditions with molecular oxygen as the electron acceptor.
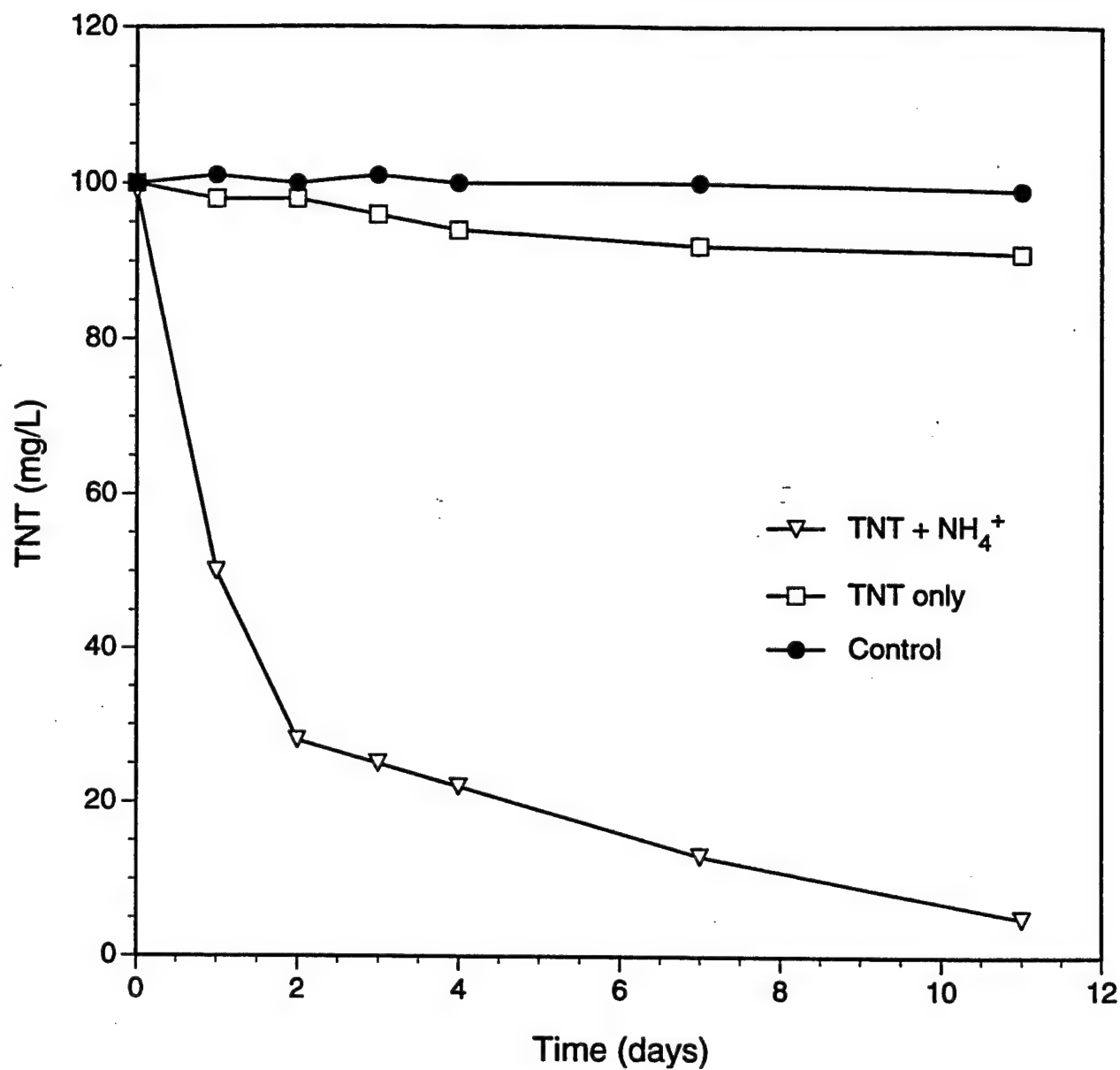
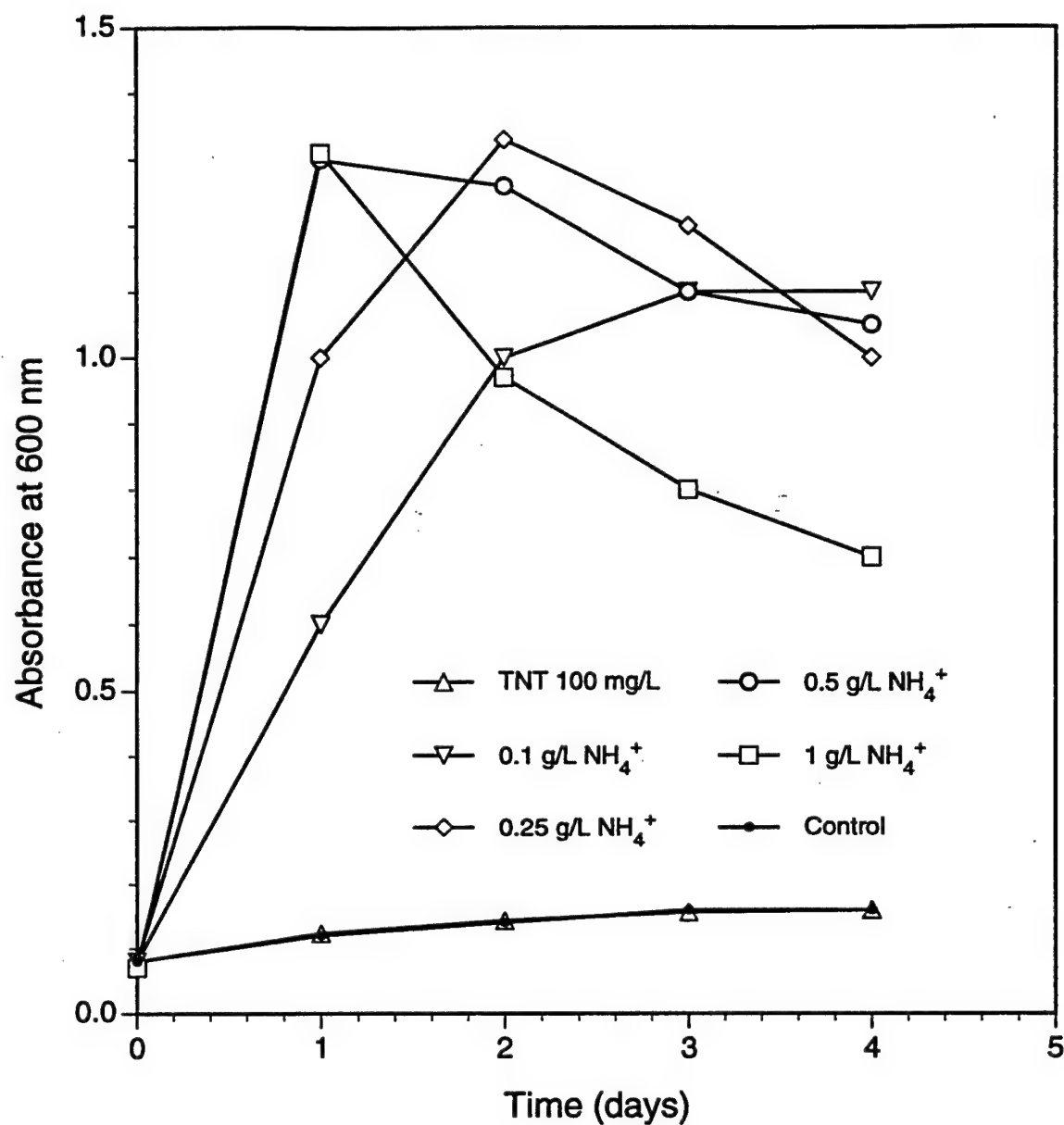FIGURE 15 TNT Metabolism in Nitrogen-Limiting Conditions

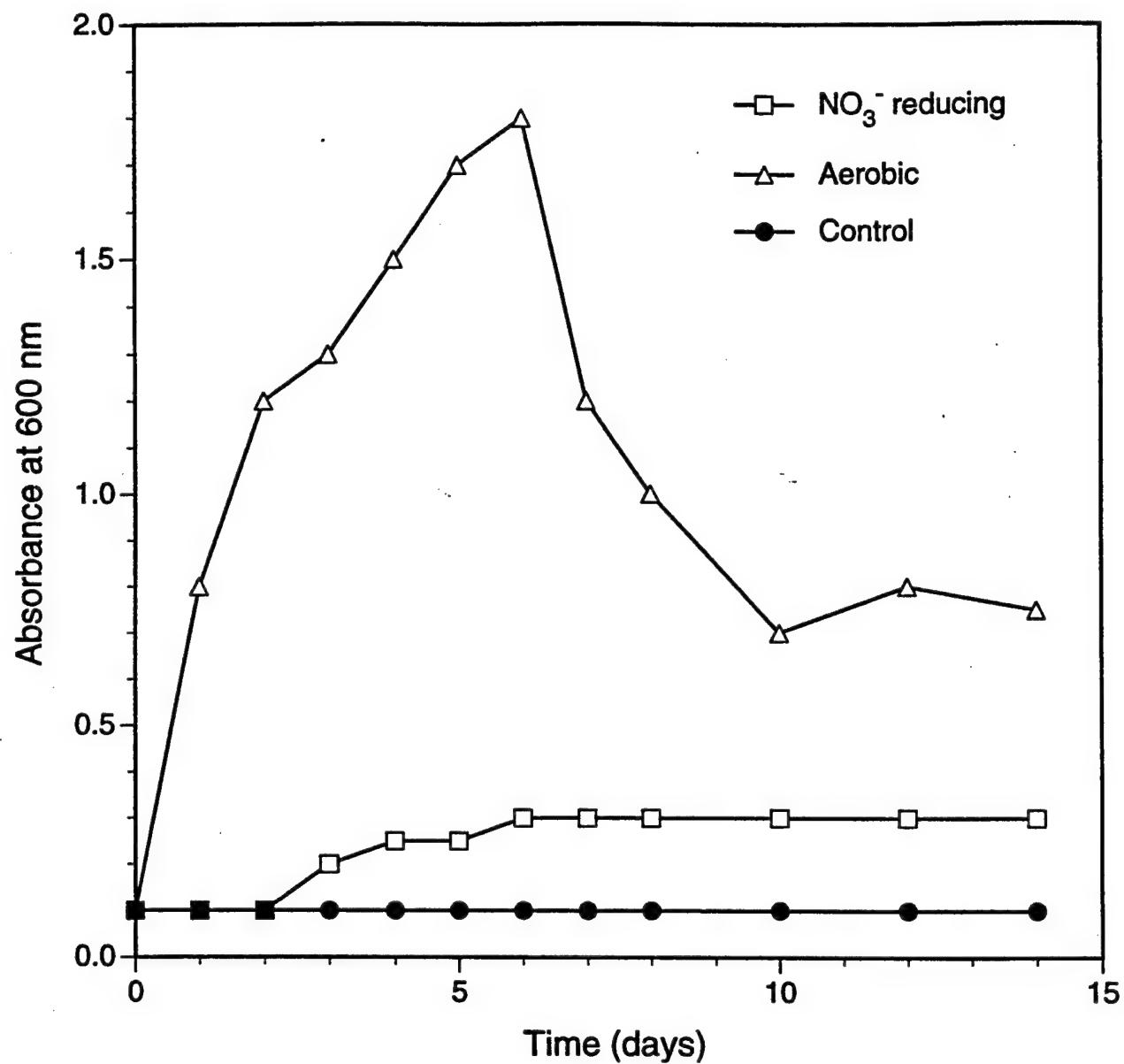FIGURE 16 Growth of RLRW with Various Nitrogen Concentrations

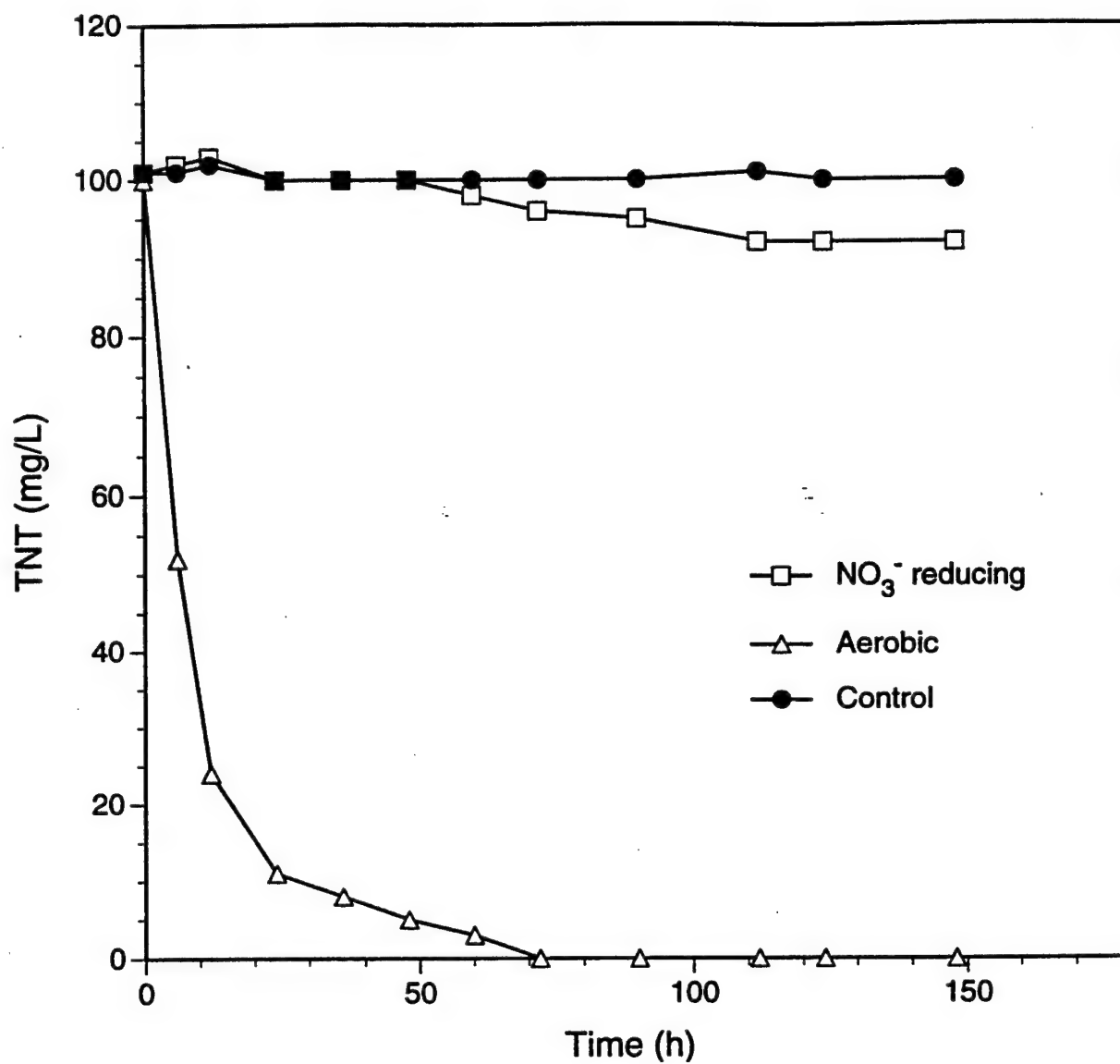FIGURE 17 Growth of RLRW under Aerobic and Nitrate-Reducing Conditions

FIGURE 18 TNT Metabolism under Aerobic and Nitrate-Reducing Conditions

5.6 Metabolism of TNT by Resting Cells and Cell-Free Extract

To determine whether TNT could be metabolized by resting cells and cell-free extract, large volumes of the RLRW consortium were cultured in a heterotrophic medium with succinate (5 g/L) as the carbon source. The results indicate that both resting cells and cell-free extract can transform TNT. The rate of TNT transformation was slower with resting cells and cell-free extract than with growing cells (Figure 19). TNT transformation was fastest with growing cells, followed by resting cells and cell-free extract. TNT transformation may occur in resting cells and cell-free extracts because of the presence of nitrite reductase enzymes, which transformed TNT.

5.7 TNT Metabolism in a Continuous Culture

To study the effect of continuous addition of TNT and substrates on the metabolism of TNT, the bacterial consortium was cultured on the heterotrophic medium with succinate (0.5%) and TNT (100 mg/L). A heat-inactivated (autoclaved) control was run in parallel for comparison. After the TNT concentration fell below 10 mg/L, 50 mg/L of TNT (5 mg in 100 mL) was added to the culture medium with succinate (2 g/L) every day. The bacterial growth and the concentration of TNT were monitored daily.

Figure 20 shows the bacterial growth in the aerobic continuous culture. The killed control, as expected, showed no growth. In the experimental flask the bacterial culture reached the stationary phase of growth after 220 h of incubation. Thereafter the culture continued to grow because of the addition of carbon source (succinate [2 g/L]). Figure 21 shows the concentration of TNT in the continuous cultures. In the control flask the TNT concentration did not decrease but increased steadily after each addition of TNT. We could not account for all of the TNT added (50 mg/L for each addition). We believe that this problem is due to the solubility of TNT in water (approximately 100 mg/L). In the experimental flask the TNT concentration fell below 10 mg/L after the ninth day of incubation. Each addition of TNT was quickly transformed by the growing bacterial cells, and after seven additions, the concentration of TNT in the culture medium was below 30 mg/L. This result proved that the bacterial consortium can remove TNT under continuous treatment, if a suitable substrate is available for the cells.

5.8 Analysis of Cell-Adsorbed TNT

To verify that the TNT was not adsorbed to the cell walls of the bacteria, bacterial cells were cultured in the presence of TNT and succinate (5 g/L). A large volume of cells (1 L) was harvested by centrifuging the cultures. The supernatant was discarded, and the cells were resuspended in methylene chloride. This extraction procedure was repeated, and the resuspended sample was analyzed by using HPLC. The results indicated that no TNT was present in the cell walls.
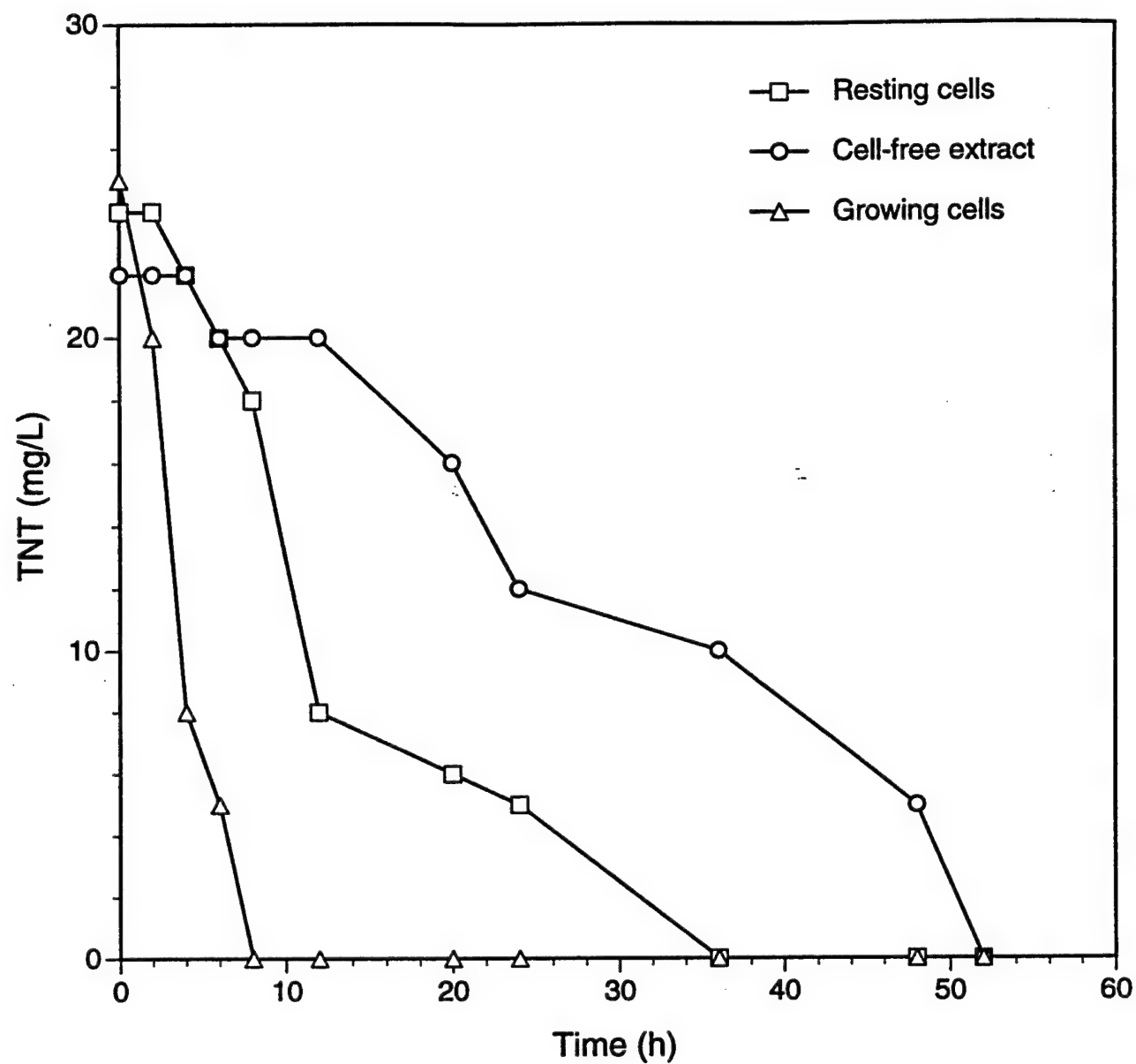
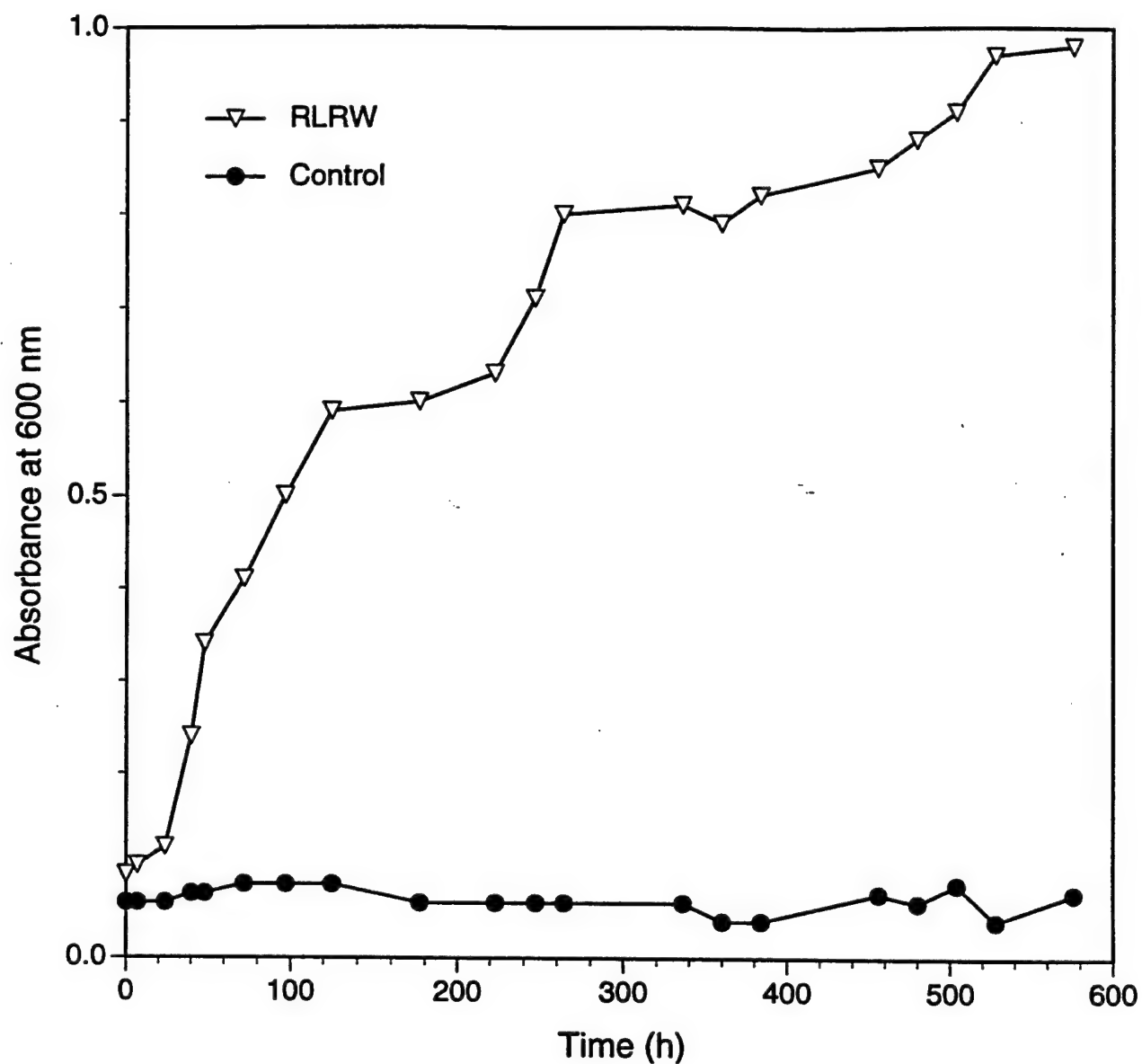FIGURE 19 Metabolism of TNT by Resting Cells and Cell-Free Extract

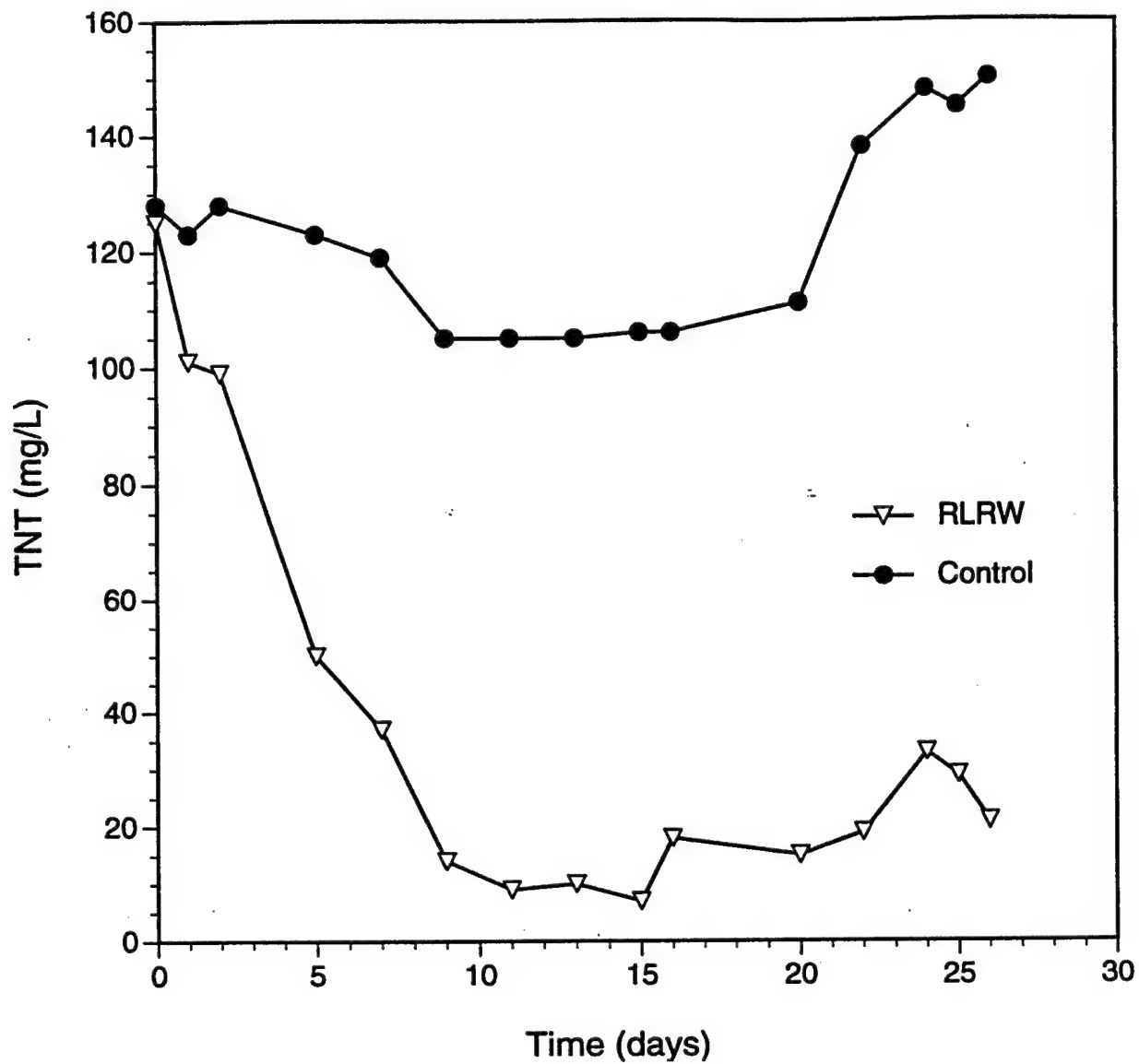FIGURE 20 Bacterial Growth in an Aerobic Continuous-Feeding System

FIGURE 21 TNT Metabolism in a Continuous Culture

To confirm these results we carried out a radiolabeling experiment. Cells were cultured in the presence of radiolabeled TNT (20,000 cpm). After growth, the cells were centrifuged, and the pellets were resuspended in a phosphate buffer. Radioactivity was measured in the resuspended cells and the supernatant. Figure 22 indicates that 86% of the radioactivity was observed in the supernatant and only 1% in the pellets.

5.9 Induction of TNT Metabolism

Purines and pyrimidines are the nitrogen bases of nucleic acids. They also act as regulatory molecules, inhibiting or stimulating the activities of certain enzymes or even controlling the overall metabolism of various compounds. In addition, purines and pyrimidines have a structural similarity to TNT. The ability of these compounds to stimulate TNT-degradation was studied.

The induction of TNT degradation in the RLRW bacterial consortium by purines and pyrimidines was studied. (Pyrimidines are cytosine, thymine, and uracil, and purines are adenine and guanine.) Cultures were pregrown in large volumes (1 L) in heterotrophic medium with 0.2% molasses and 0.05% each of the purine or pyrimidine bases. Cultures were centrifuged, and the cells were resuspended in heterotrophic medium with TNT (100 mg/L) as the sole source of carbon and energy. The experiment was conducted in duplicate. Controls without purine- or pyrimidine-induced cells and with killed (heat-inactivated) cells were used. The concentration of TNT was analyzed daily.

Figures 23 and 24 show that no induction of the TNT degradation process occurred. The TNT concentration dropped even with the uninduced cells because of the large cell numbers. Cultures with 0.1% and 0.5% inocula of purine/pyrimidine-induced cells showed no degradation of TNT, indicating that purines and pyrimidines do not induce TNT degradation by this bacterial consortium.

5.10 Studies with Pure Cultures

Four aerobic bacteria were isolated from the soil contaminated with TNT. All four isolates were gram-negative rods identified as *Pseudomonas* spp. (Table 3). Figure 25 shows the growth curves for the isolates. The isolates were cultured in heterotrophic medium with 0.5% succinate (5 g/L) and TNT (100 mg/L). Isolates 1 and 2 grew rapidly and reached a maximum optical density of 1.10 at 600 nm within 31 h of incubation. Isolate 4 grew slowly, with a lag period of 24 h, and reached maximal growth after 140 h of incubation. The TNT concentration dropped rapidly with all four isolates. TNT was completely transformed within four days of incubation by isolate 4. Isolates 1, 2, and 3 needed eight days to transform 100% of the TNT (Figure 26).
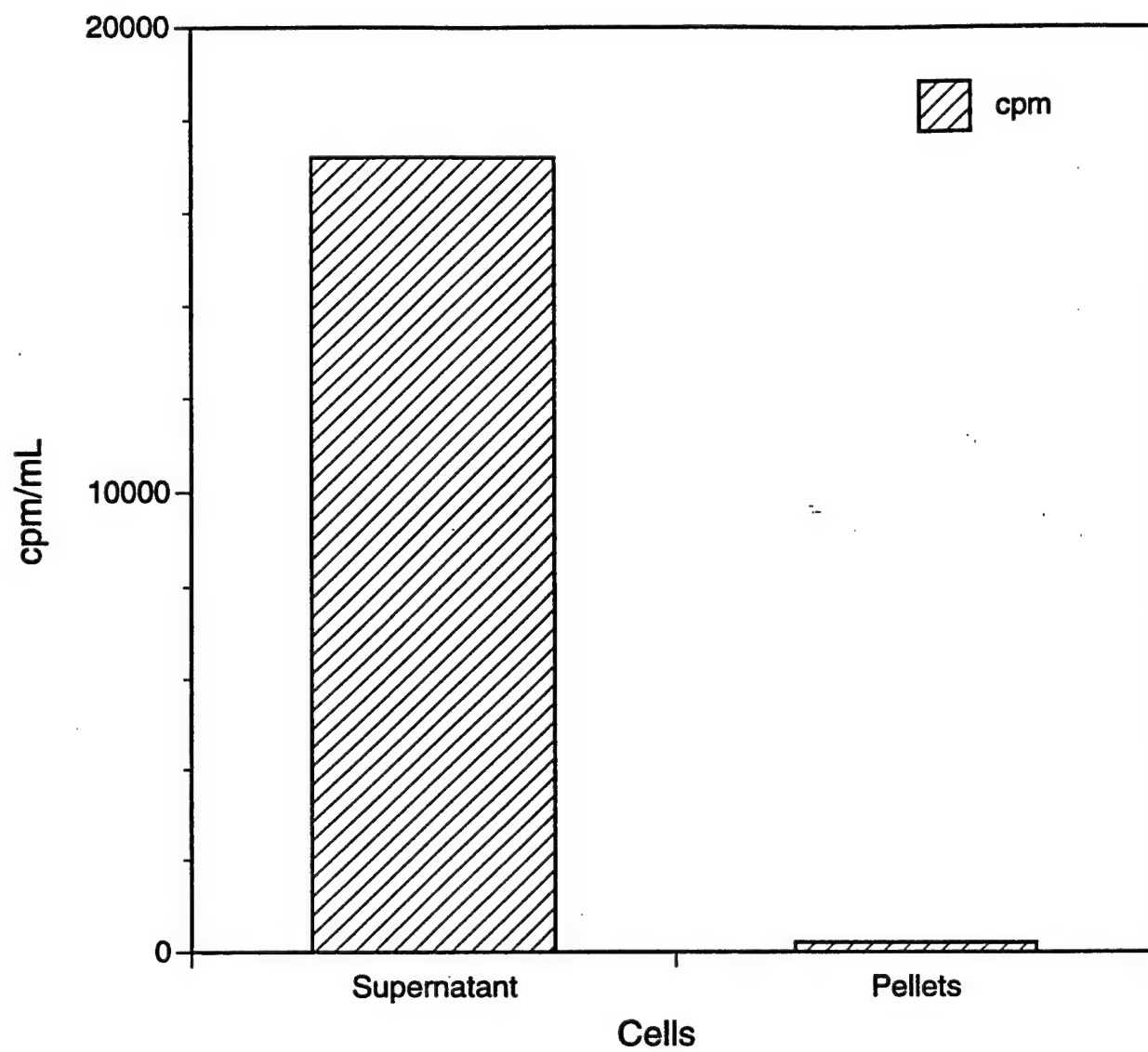
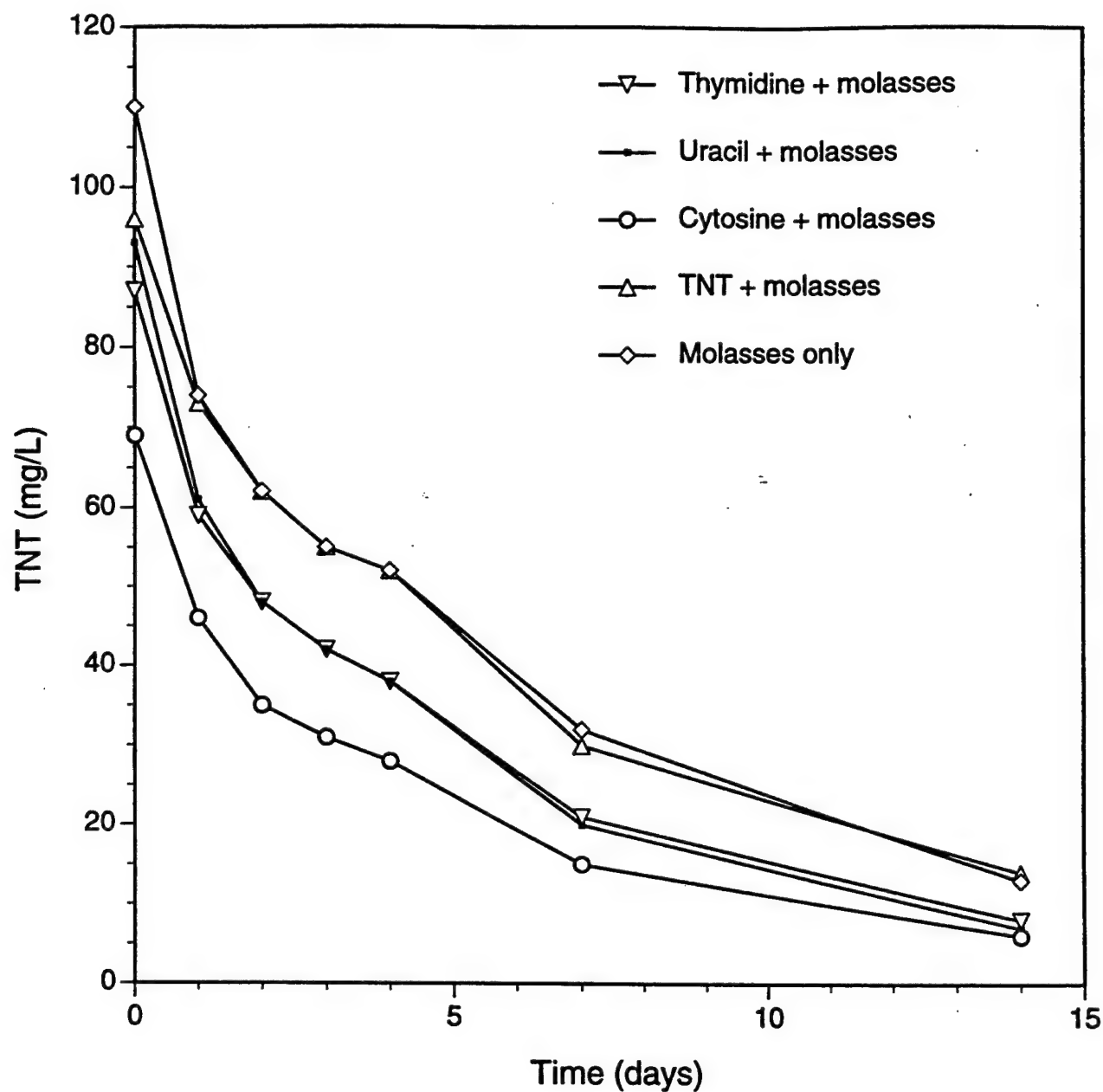FIGURE 22 Radioactivity in Cells and Supernatant

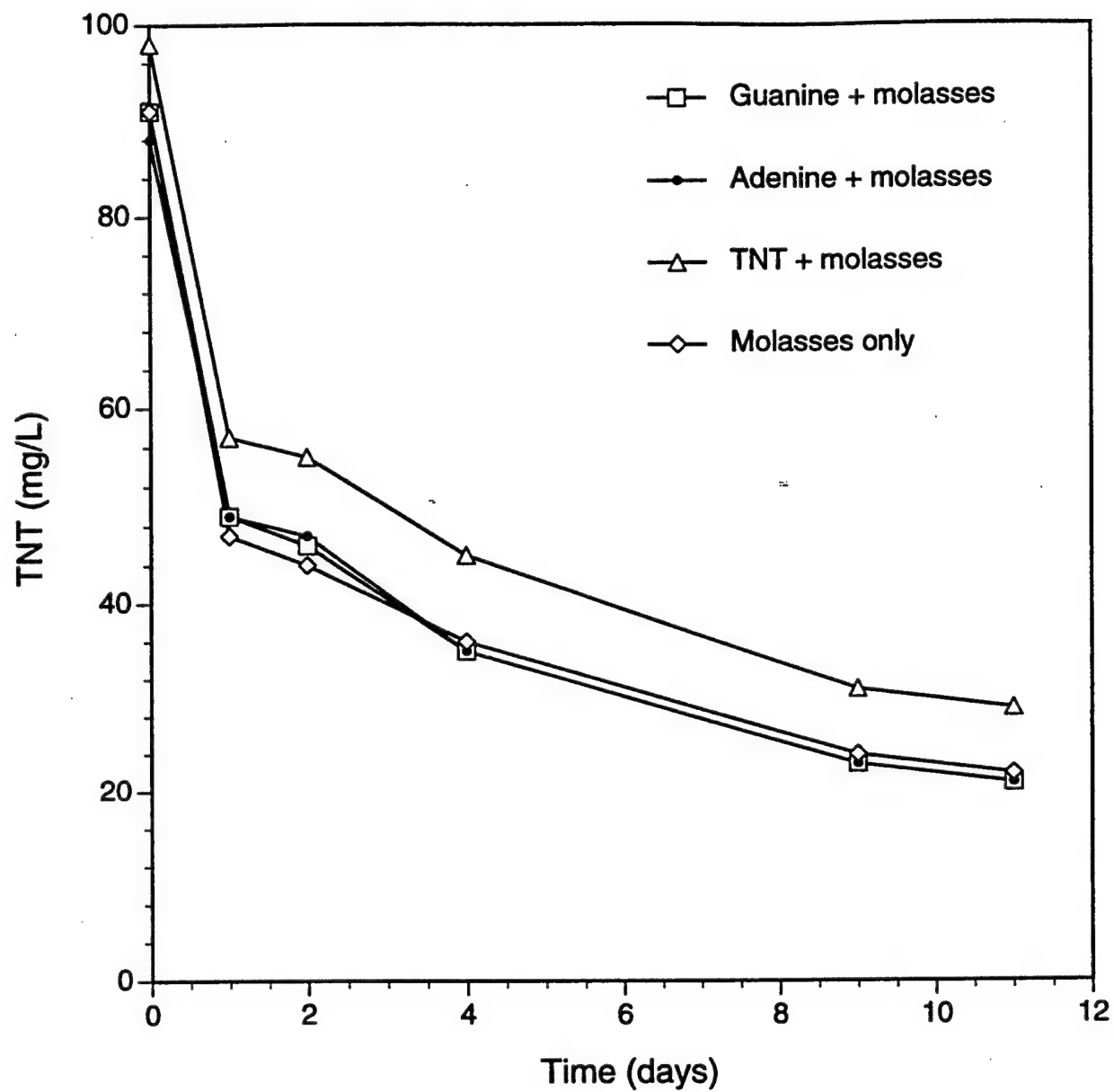FIGURE 23 Effect of Pyrimidine-Induced Cells on TNT Metabolism

FIGURE 24 Effect of Purine-Induced Cells on TNT Metabolism

TABLE 3 Pure Cultures Isolated from the Soil Consortium

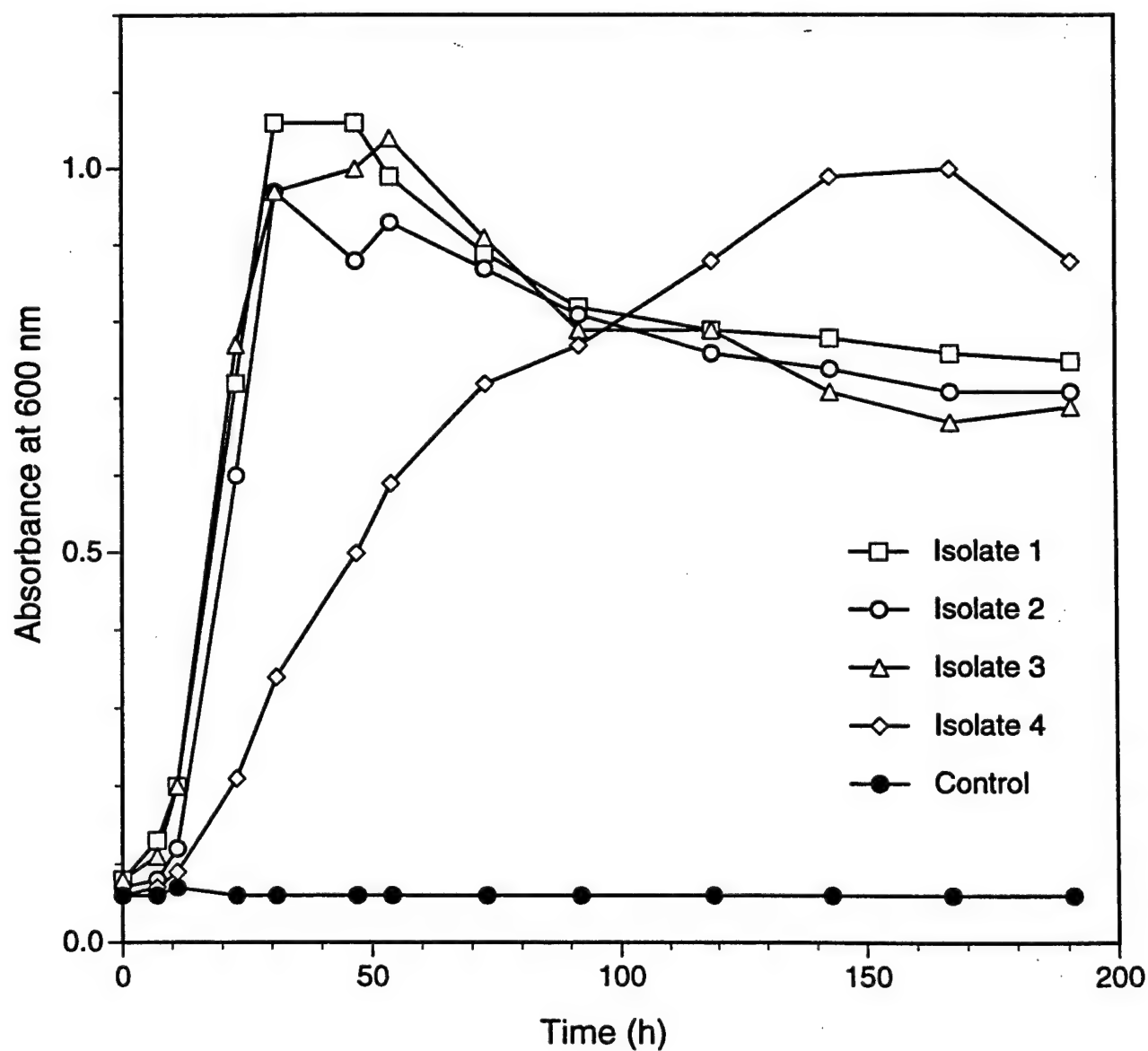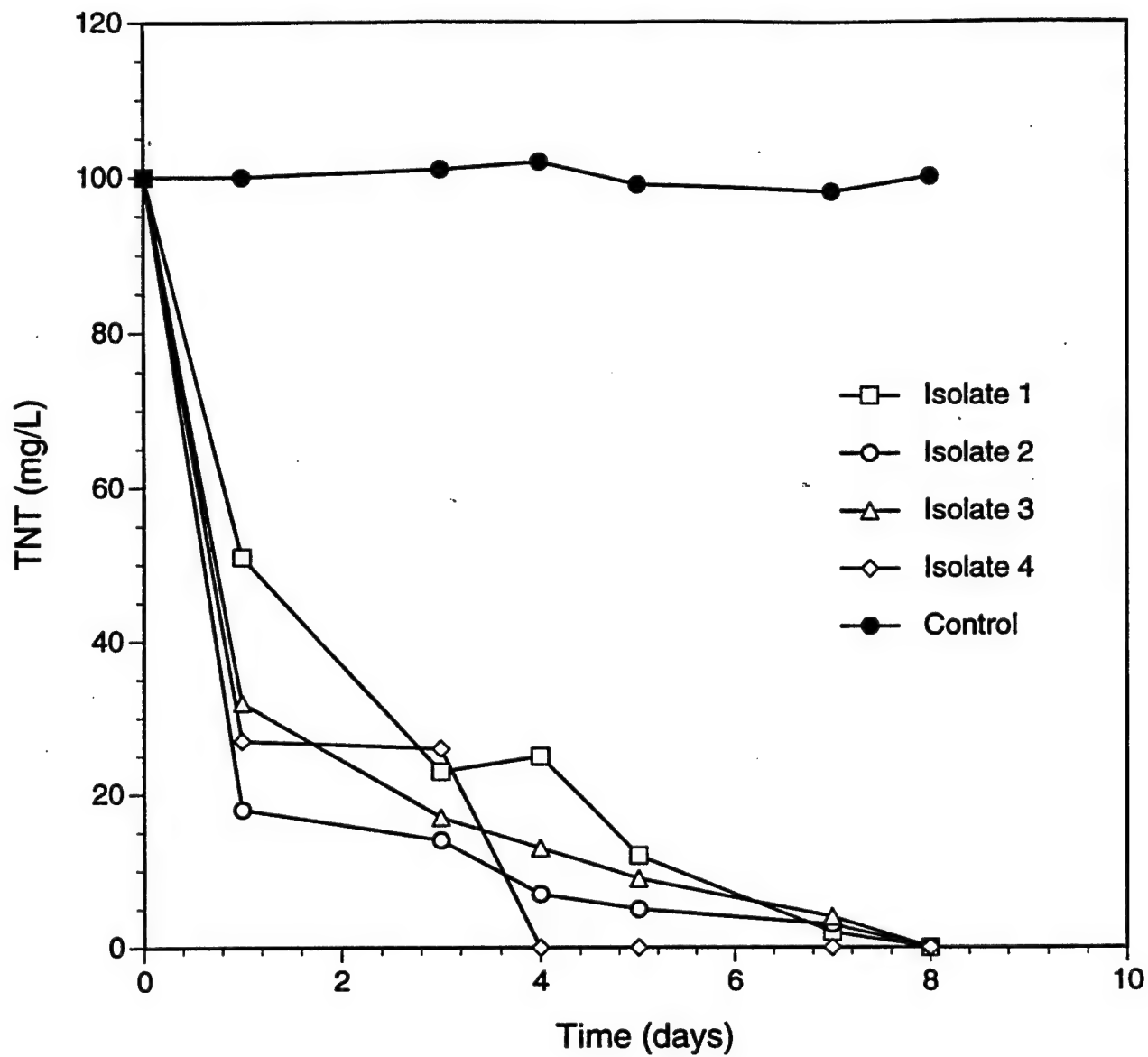| Isolate	Organism
1	*Pseudomonas acidovorans*
2	*Pseudomonas fluorescens*
3	*Pseudomonas mendocina*
4	*Pseudomonas aeruginosa*

FIGURE 25 Growth of Different Isolates

FIGURE 26 Metabolism of TNT by Different Isolates

In the cultures with succinate, the nitrite concentration in the culture medium increased during incubation. The nitrite concentration was high for isolate 1, with a maximum of 1,800 µg/L (equal to 11% of the nitrogen present in TNT) observed on the 11th day of incubation. Isolate 3 showed a similar trend. Isolate 4 produced considerably less nitrite (Figure 27).

Radiolabeling experiments showed that isolate 3 used 13% of the [^{14}C]TNT to make cellular material. Isolates 1 and 2 converted about 8% of the [^{14}C]TNT to biomass, while isolate 4 converted about 6% of the [^{14}C]TNT to TCA-precipitable material (Figure 28). Analysis of the methylene chloride extracts of the cells revealed that no TNT was bound to the cell walls of the bacteria. Incubation of isolate 4 up to 14 days resulted in ring cleavage; approximately 1% of [^{14}C]TNT was converted to $^{14}CO_2$. For the other isolates, 0.60% of [^{14}C]TNT was converted to $^{14}CO_2$ (Figure 29).

The HPLC analysis of the four-day-old cultures showed that [^{14}C]TNT was converted to intermediates. On the HPLC elution profile of authentic TNT, [^{14}C]TNT was eluted at 7 min. Fractions were collected every minute, and radioactivity was measured by using a scintillation counter. Analysis of the isolate 4 culture revealed two intermediates that eluted before TNT, the first at 2 min and the second at about 5 min (Figure 30). Similar intermediates were observed for the other isolates. Heat-inactivated controls and the culture without succinate produced no intermediates, and the TNT concentration remained at the original level throughout the incubation period.

The GC/MS analysis of the intermediates clearly showed that three different metabolites were produced from TNT. Two intermediates were identified as 4-amino-2,6-dinitrotoluene and its isomer, 2-amino-4,6-dinitrotoluene. The third intermediate was not identified. The results of mass balance analysis of the culture sample showed that 85% of the TNT was found in the intermediates, while 6-13% was converted to cell biomass, and < 1% was evolved as $^{14}CO_2$.

This pure culture experiment clearly showed that each member of the consortium has a specific function. For example, isolate 1 produced more nitrite from TNT than isolate 4, and isolate 3 used more TNT to make biomass than other isolates.

5.11   Studies of Aerobic/Anoxic Soil Slurry Reactor Operation

With the information gained from the previous experiments, it was possible to follow the degradation pattern of TNT in soil slurries. Semibatch soil slurry reactors were simulated in 0.5-L laboratory-scale aerobic/anoxic reactors, and the degradation of TNT was monitored along with other parameters. TNT in the soil was extracted with acetonitrile. The eluent for HPLC analysis was methanol:water at 50:50 V/V.
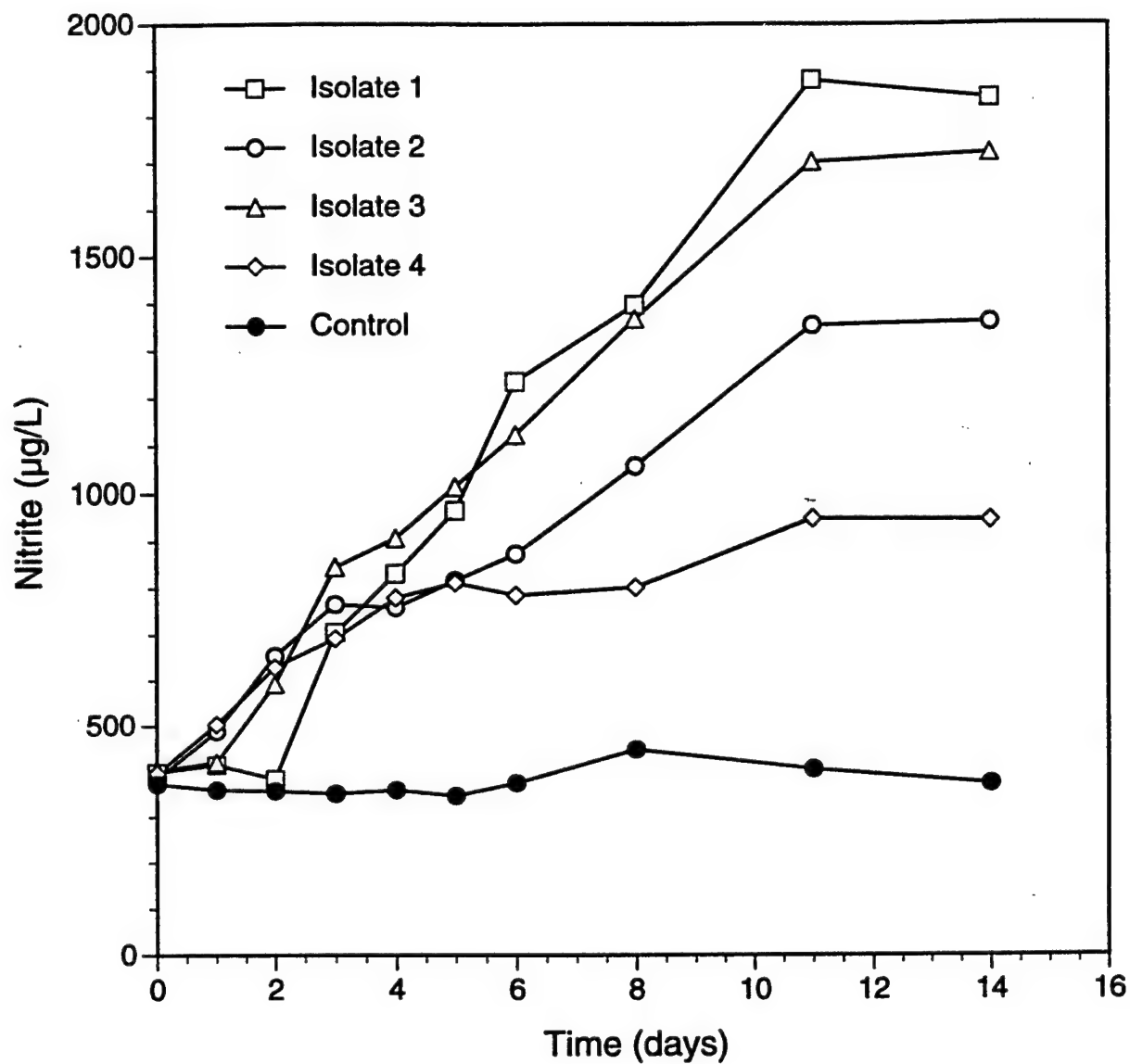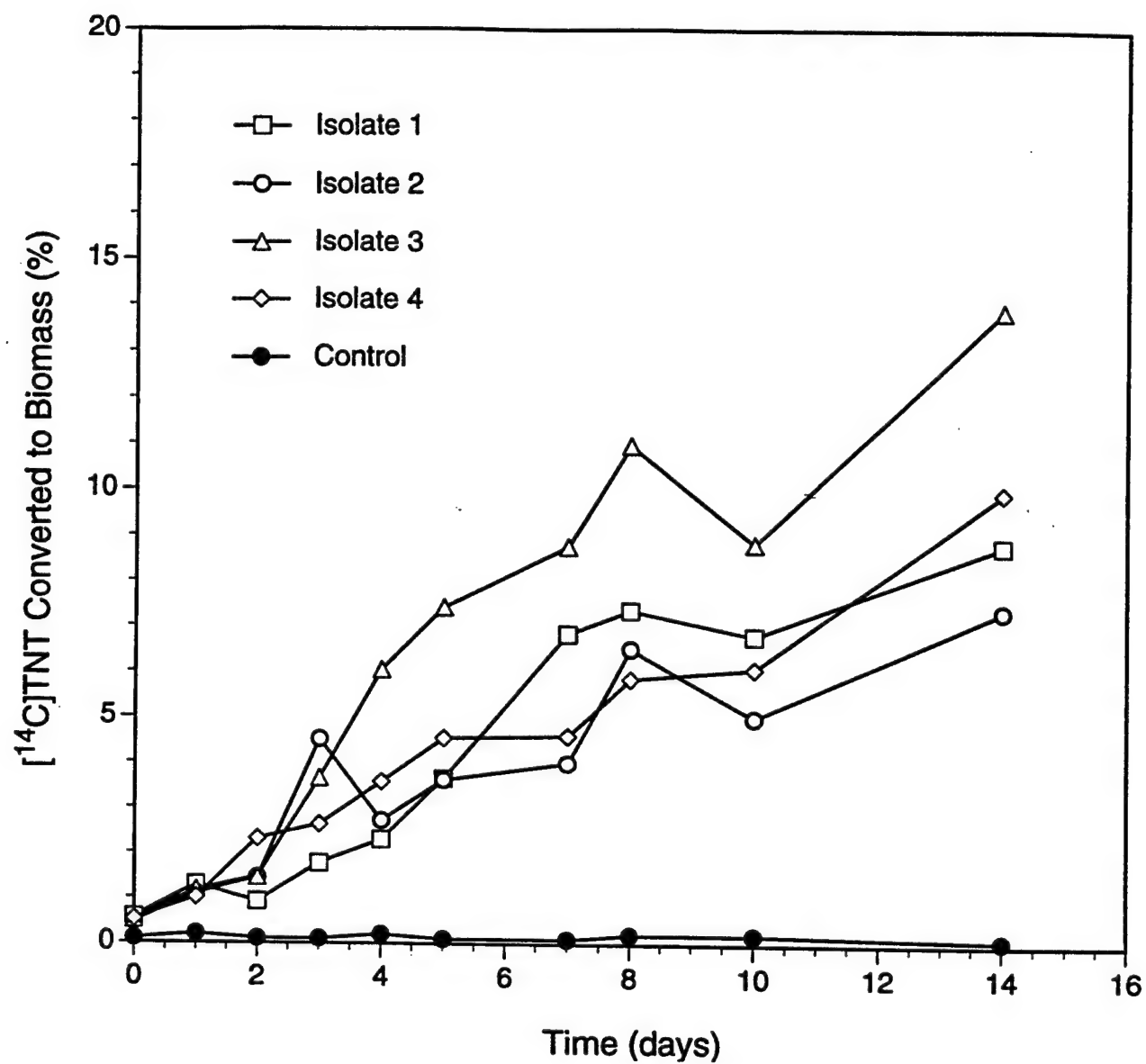
FIGURE 27   Nitrite Concentration for Different Isolates

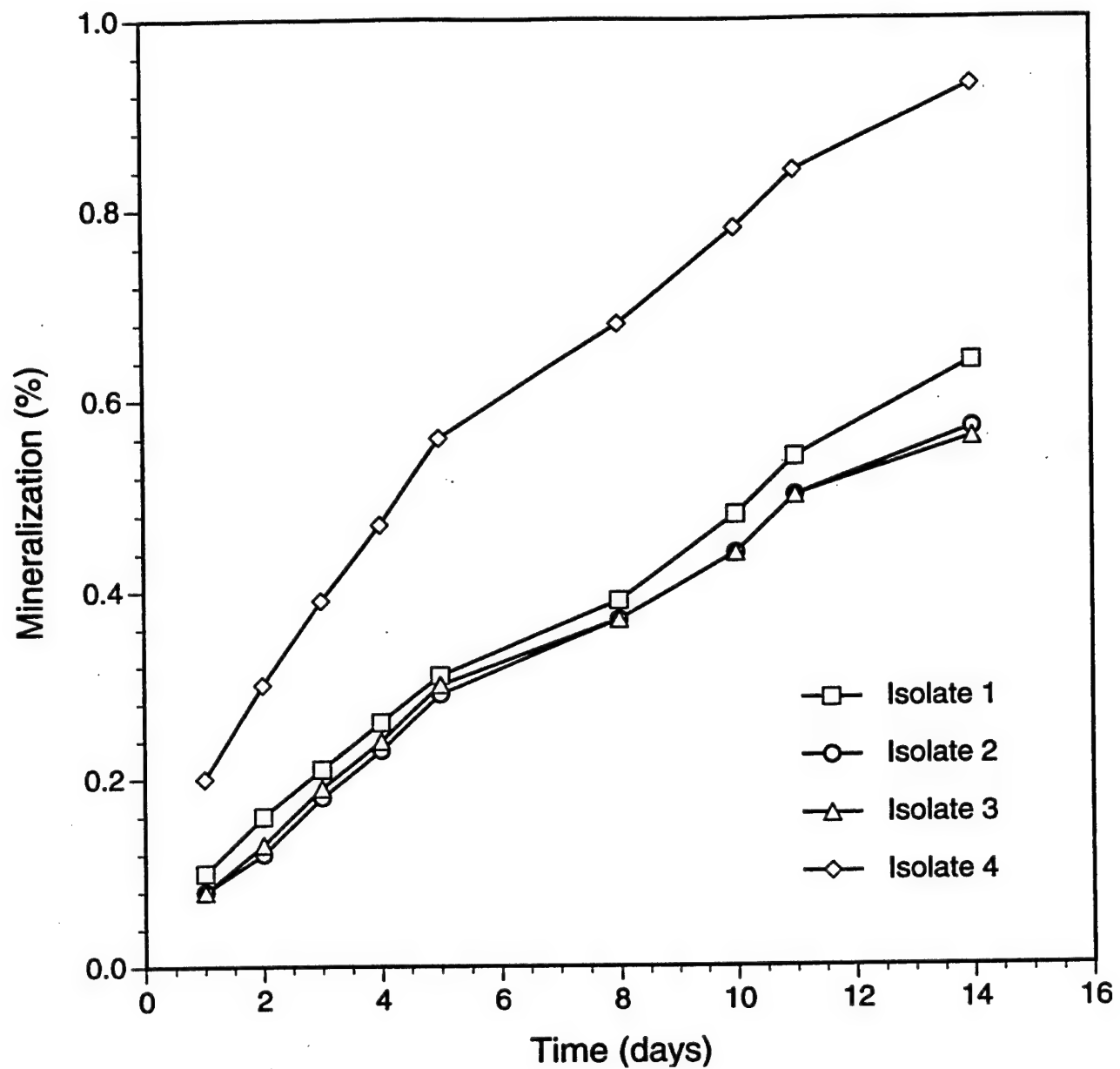FIGURE 28 [^{14}C]TNT Converted to Biomass by Different Isolates

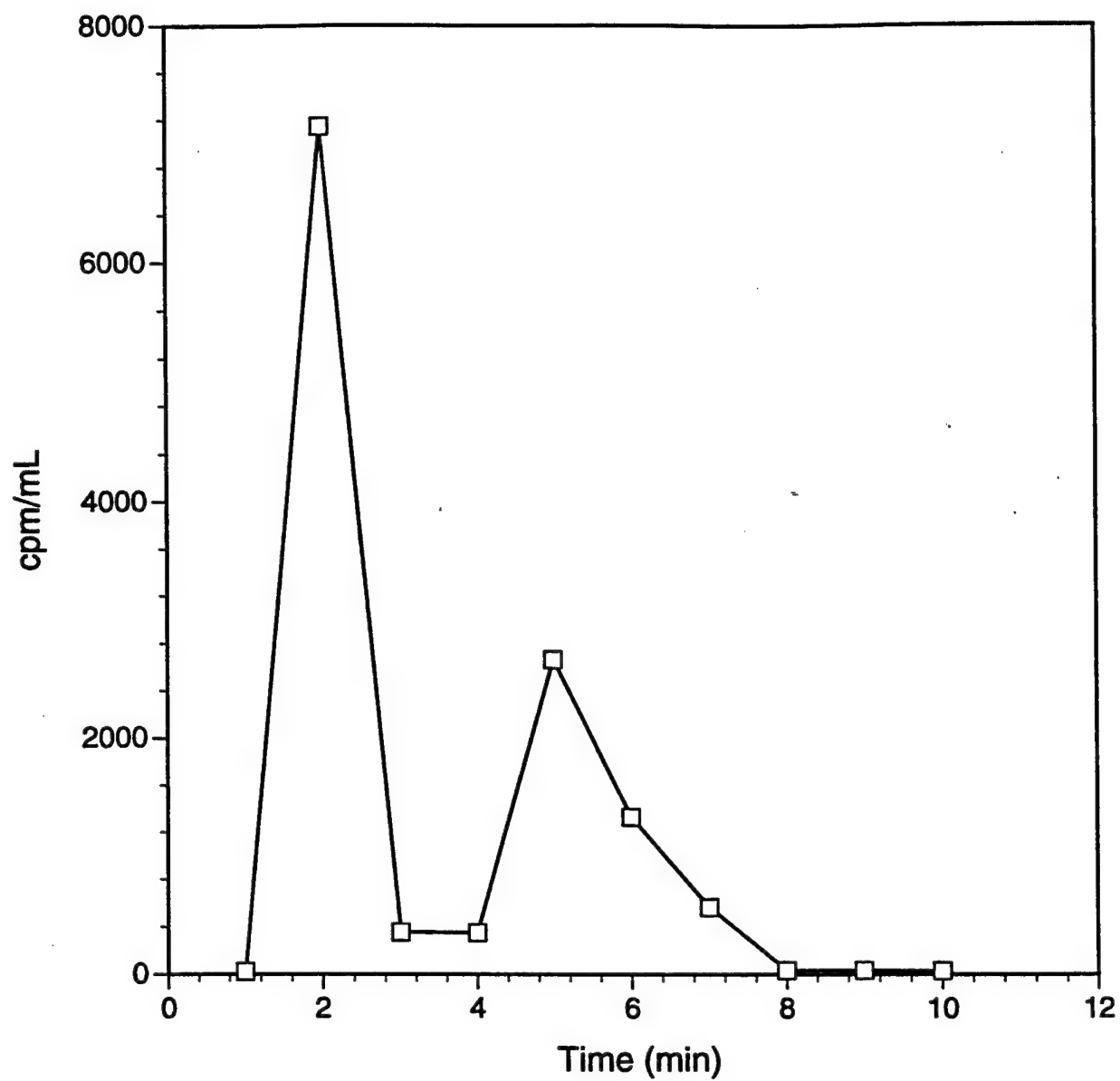FIGURE 29 [^{14}C]TNT Converted to $^{14}CO_2$ by Different Isolates

FIGURE 30 HPLC Elution Profile of Isolate 4

5.11.1   Soils

Contaminated soil was collected at the Joliet Army Ammunition Plant, Joliet, Illinois. The contaminant concentrations in the soil are given in Table 1. The TNT concentration in the soil ranged from 10,000 to 20,000 mg/kg. The concentrations of the other contaminants, RDX, HMX, TNB, and DNT, in soil were less than 300 mg/kg.

5.11.2   Soil Slurry Reactor

Two 0.5-L laboratory-scale soil slurry reactors were set up as shown in Figure 1. The reactors were operated in a semibatch mode. The reactors were started with 15% (W/V) of TNT-contaminated soil. Molasses (0.3%) served as the carbon source. Nitrogen in the form of ammonium chloride (0.1 g/L) was added to both reactors. Air was supplied through a diffuser for 15-30 min each day. The soil slurry was mixed continuously with a magnetic stirrer. The concentration of dissolved oxygen in the biologically active reactor and the rate of reaeration of the deaerated water (without biomass) are shown in Figures 31 and 32, respectively. After a two-week stabilization period, 10% of the contaminated soil was replaced weekly along with molasses (0.3%) and ammonium chloride (0.1 g/L). After 200 days of operation, the soil replacement was increased to two times a week (with 10% soil replacement each time) in one reactor and three times a week (with 10% soil replacement each time) in the other reactor. Carbon (molasses) and inorganic nitrogen (ammonium chloride) were added along with the soil to the reactor. The concentrations of TNT and other contaminants, nitrite, and ammonia; bacterial growth; pH; and oxygen uptake were monitored periodically.

5.11.3   Performance of the Reactor

The distribution of TNT in the soil at Joliet is not homogeneous. Some parts of the contaminated site have high TNT concentrations, whereas other parts have lower concentrations. This could explain why the TNT concentration at the beginning of the experiment was so high (nearly 8,000 mg/kg) (Figure 33). The soil used as replacement material generally had a TNT concentration of 6,000-8,000 mg/kg. The concentration of TNT and its metabolites 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene are given in Figure 33.

The first two weeks of reactor operation were in the typical batch mode, and the concentration of TNT in the reactor dropped during this period. On day 15 the level of TNT was less than 1,000 mg/kg. After two weeks of operation, a 10% volume of contaminated soil was added to the reactor. Thereafter 10% of the soil slurry was replaced every week with contaminated soil. Whenever soil was added, the concentration of TNT increased, then decreased on the next day. This rise and fall of TNT levels was seen throughout the first two months of reactor
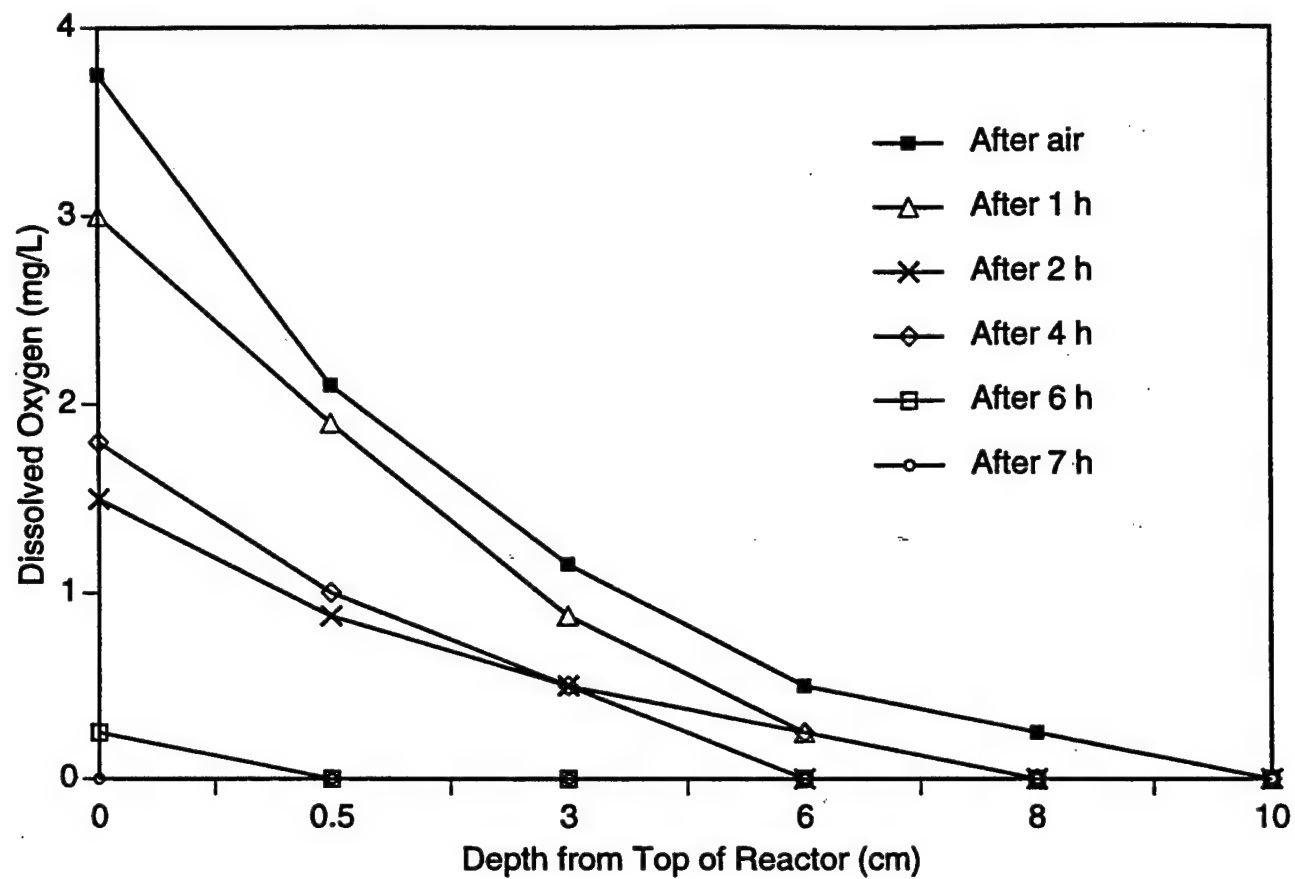
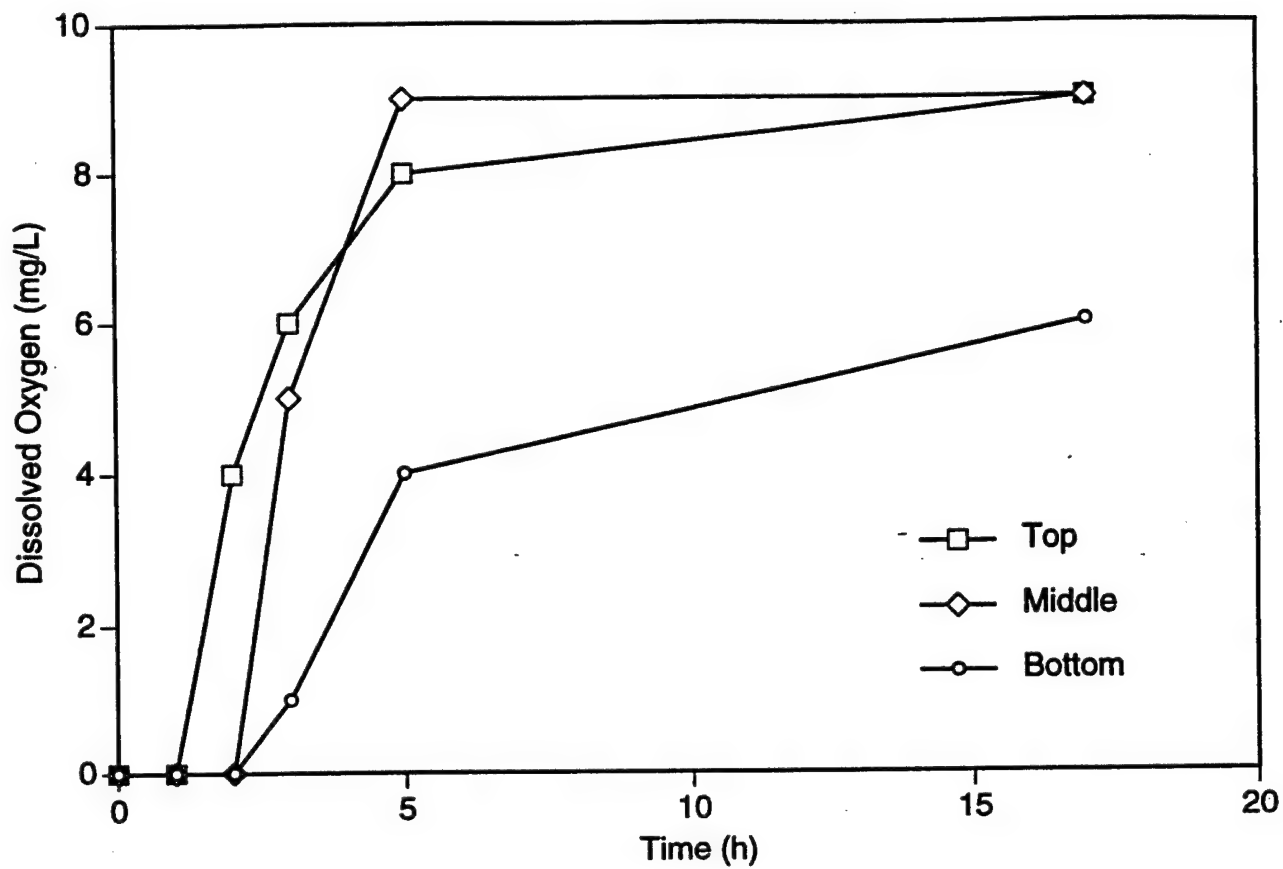FIGURE 31 Dissolved Oxygen in the Reactor

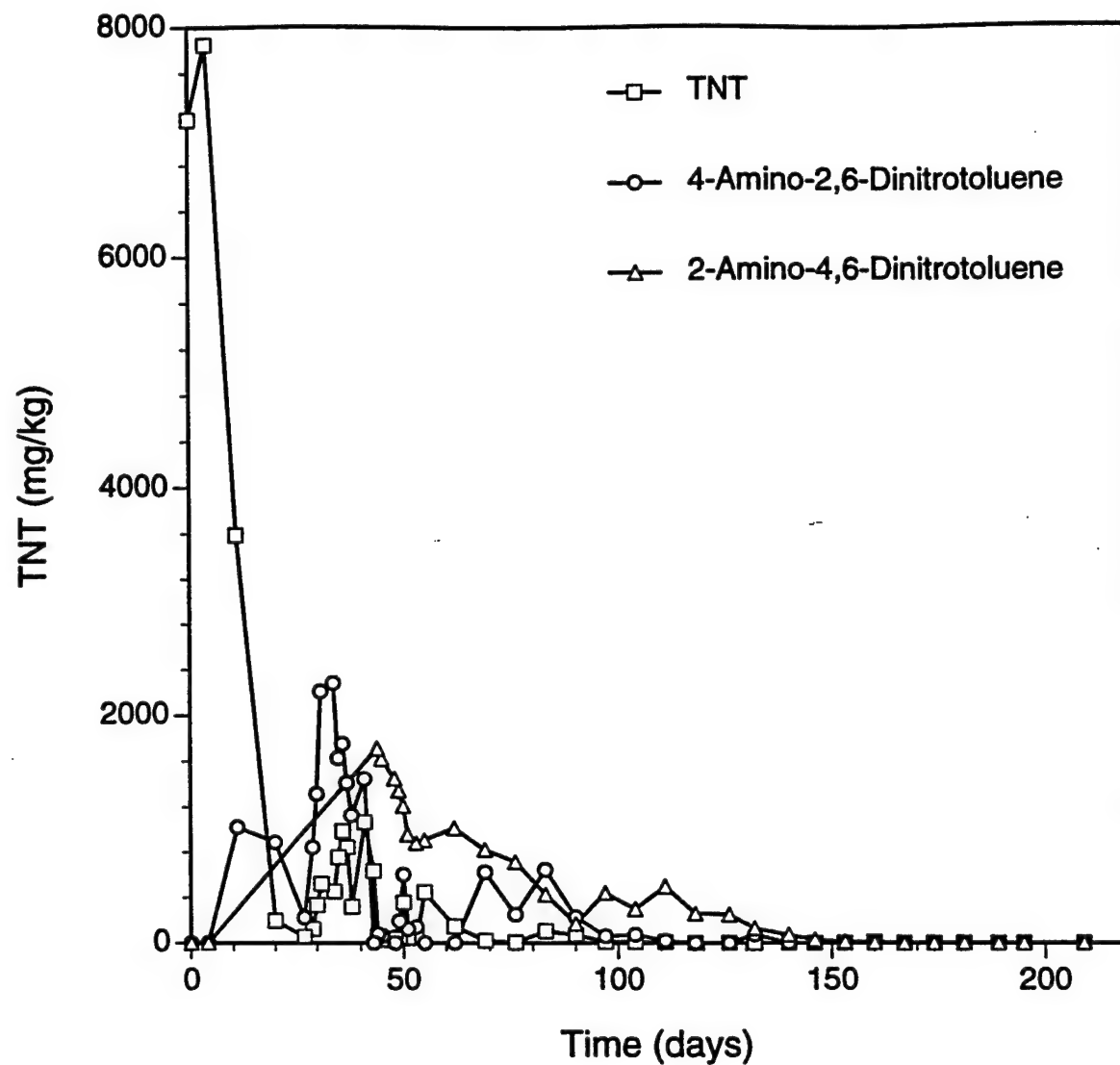FIGURE 32 Reaeration of the Laboratory Reactor

FIGURE 33 Concentrations of TNT and Its Metabolites in the Reactor

operation. After two months of operation, the TNT concentration in soil dropped steadily, eventually falling below the detection limit (0.5 mg/kg) on the 95th day. The concentrations of the 4-amino and 2-amino TNT metabolites were also monitored throughout the reactor operation. As Figure 33 shows, the concentrations of intermediates ranged from 0 to 2,100 mg/kg, generally increasing upon soil addition and decreasing the next day. After four months of continuous operation, the concentration of TNT metabolites was less than 20 mg/kg of soil.

Figure 34 shows the concentrations of the munitions compounds present in the soil. The TNB concentration dropped to 0 mg/kg from 300 mg/kg within 50 days. The next compound to completely disappear from the slurry system was 2,4-DNT. The RDX and HMX persisted in the soil longer than the other compounds. No drop in RDX and HMX concentrations occurred during the first 30 days of reactor operation, but then the concentrations of both compounds gradually decreased. After 120 days of continuous operation of the slurry reactor, the concentrations of RDX and HMX fell below 0.5 mg/kg of soil. Even though the concentrations of these compounds are relatively insignificant, their removal from the soil enhances the bioremediation potential of the soil slurry reactor system.

The bacterial activity in the reactor was monitored by using total plate counts on TSA plates. The counts in the reactor were in the range of 10^6 to 10^7 CFU/mL (colony-forming units per milliliter) at the beginning, and the counts increased steadily throughout the reactor operation. A slight fluctuation in colony counts was due to the weekly addition of soil, carbon, and nitrogen to the reactor. During most of the reactor operation the total counts were about 10^8-10^9 CFU/mL (Figure 35). No significant drop in colony counts occurred throughout the study.

Figure 36 shows the pH and oxygen uptake in the reactor. The initial pH of the soil slurry was 7.5. During the first two weeks of operation the pH was not adjusted. Later, whenever the pH dropped below 6.5 or rose above 7.5, the pH was adjusted to 7 by using 0.1 N HCl or 0.1 N NaOH. No serious drop in the pH level occurred in either reactor up to 150 days of operation, but the pH was acidic (close to 6) after 150 days of reactor operation. The oxygen uptake rate of reactor biomass increased from day 0 and then was maintained at about 7.5 mg/L·h throughout the experimental period. This result suggests that the biomass in the reactor was very active and that no identifiable toxic effects on the soil microbes occurred from either the TNT or the TNT metabolites.

The radiolabeling studies with the reactor biomass demonstrated the mineralization of TNT (Figure 37). The $^{14}CO_2$ increased gradually from 0.1% on day 0 to 23% on day 14. In the killed control, less than 0.3% $^{14}CO_2$ was observed. This observation clearly demonstrates that the TNT was mineralized by the soil bacteria in the soil slurry system, and it explains the disappearance of the 4-amino and 2-amino TNT metabolites from the reactor. The analysis of TCA-precipitable material showed that a significant amount of [^{14}C]TNT was converted to cell biomass. The radioactivity in TCA-precipitable material increased gradually from day 0 to day 14. The maximum
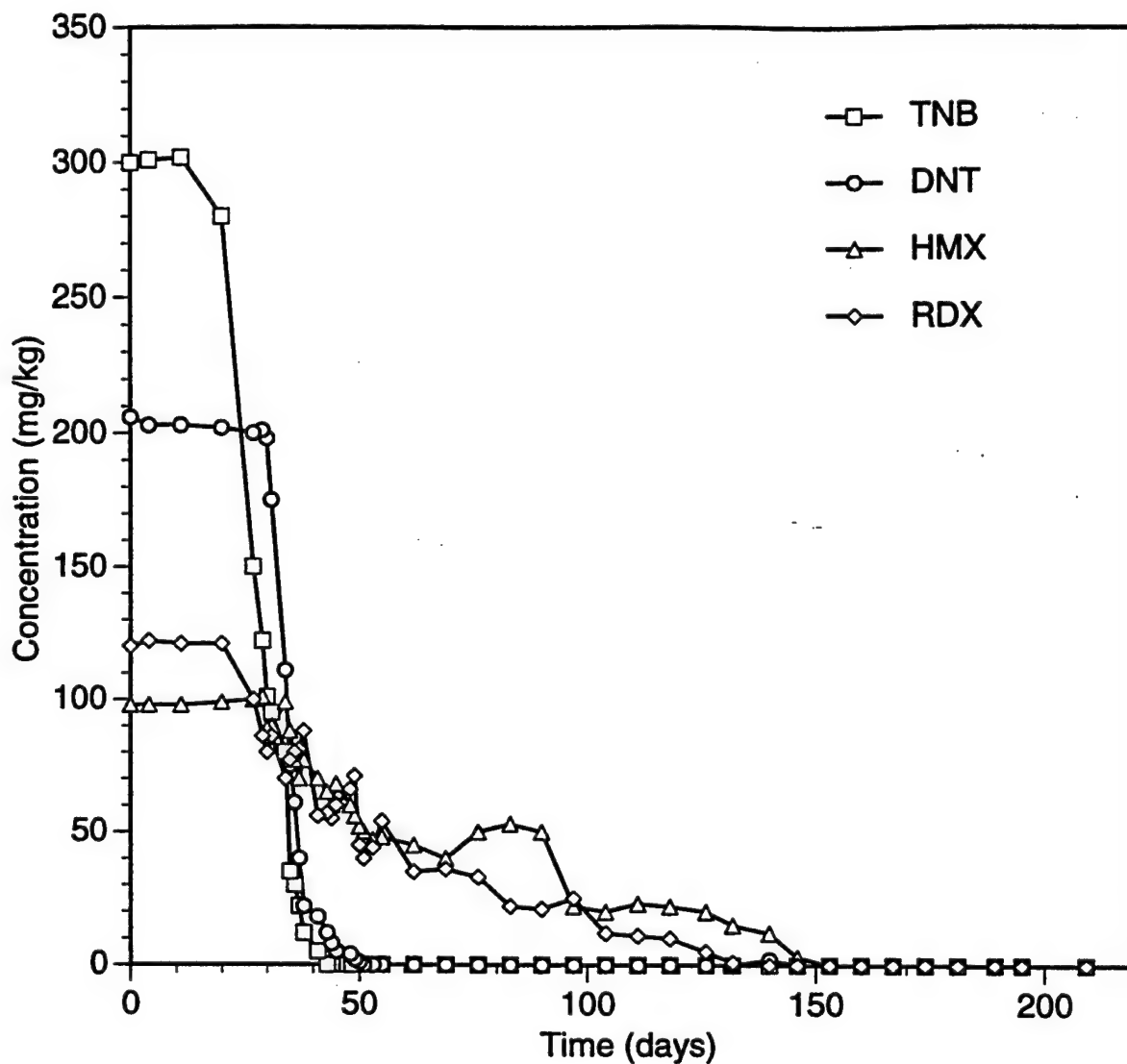
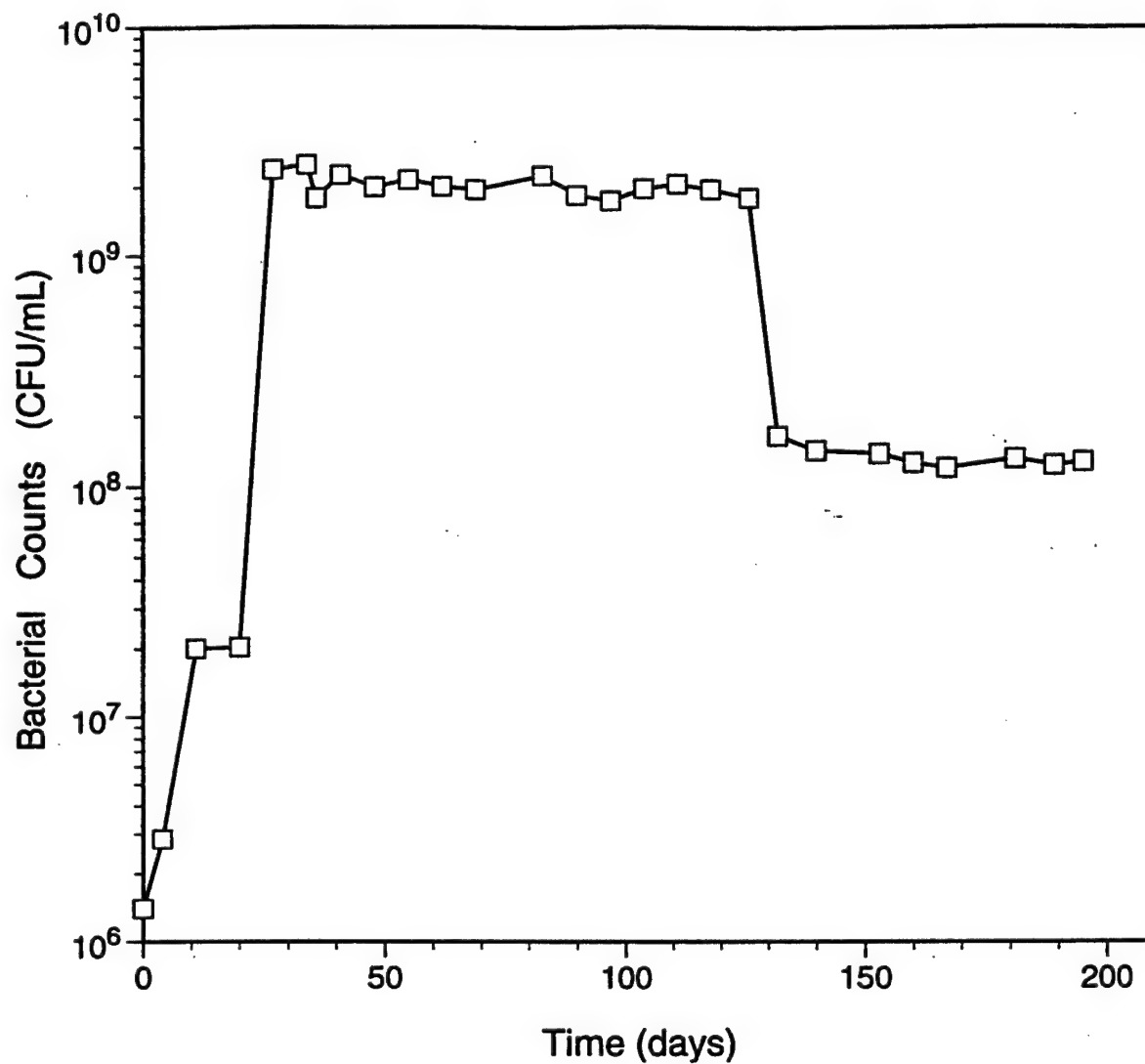FIGURE 34 Concentrations of Munitions Compounds in the Reactor
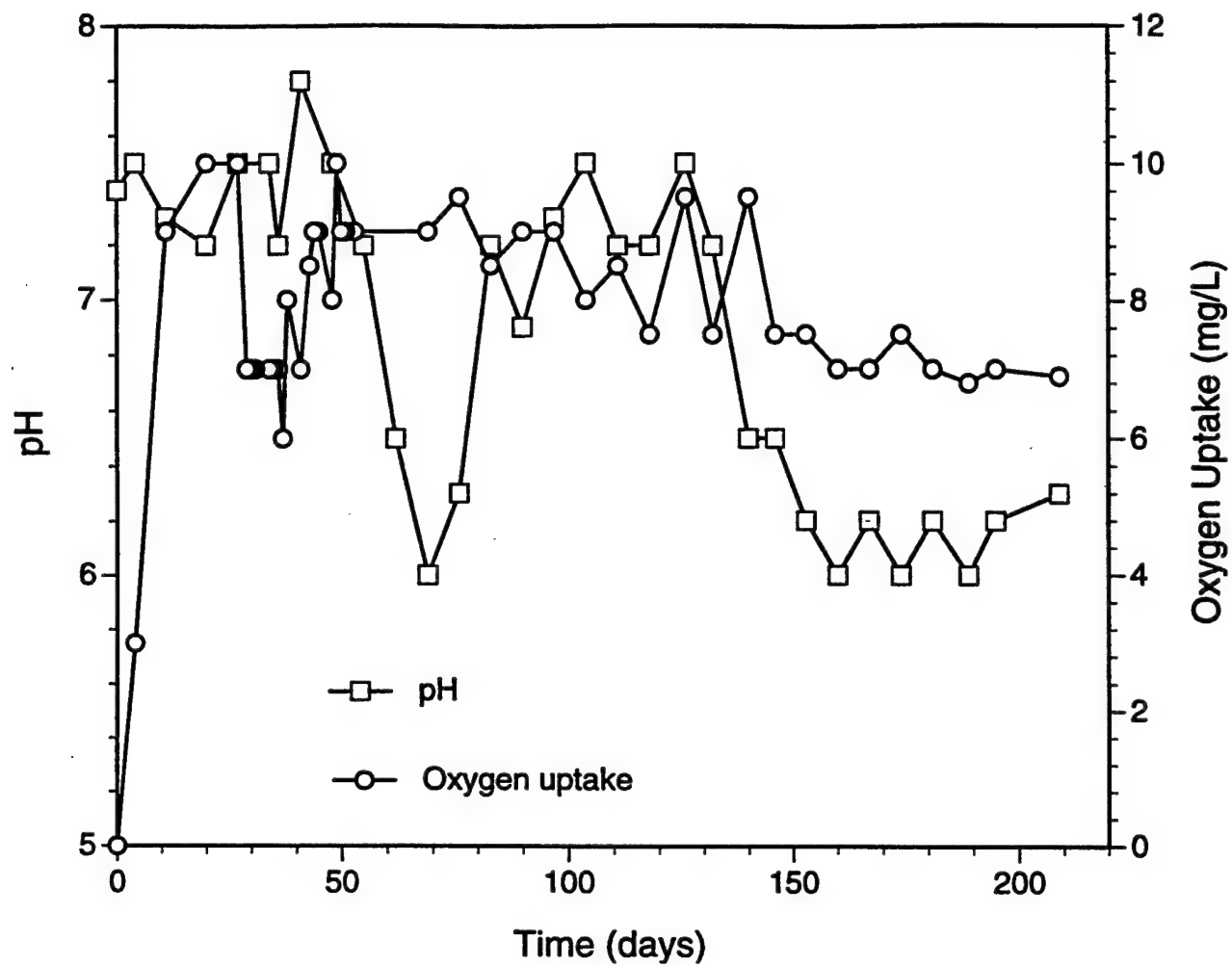
FIGURE 35 Bacterial Counts in the Reactor

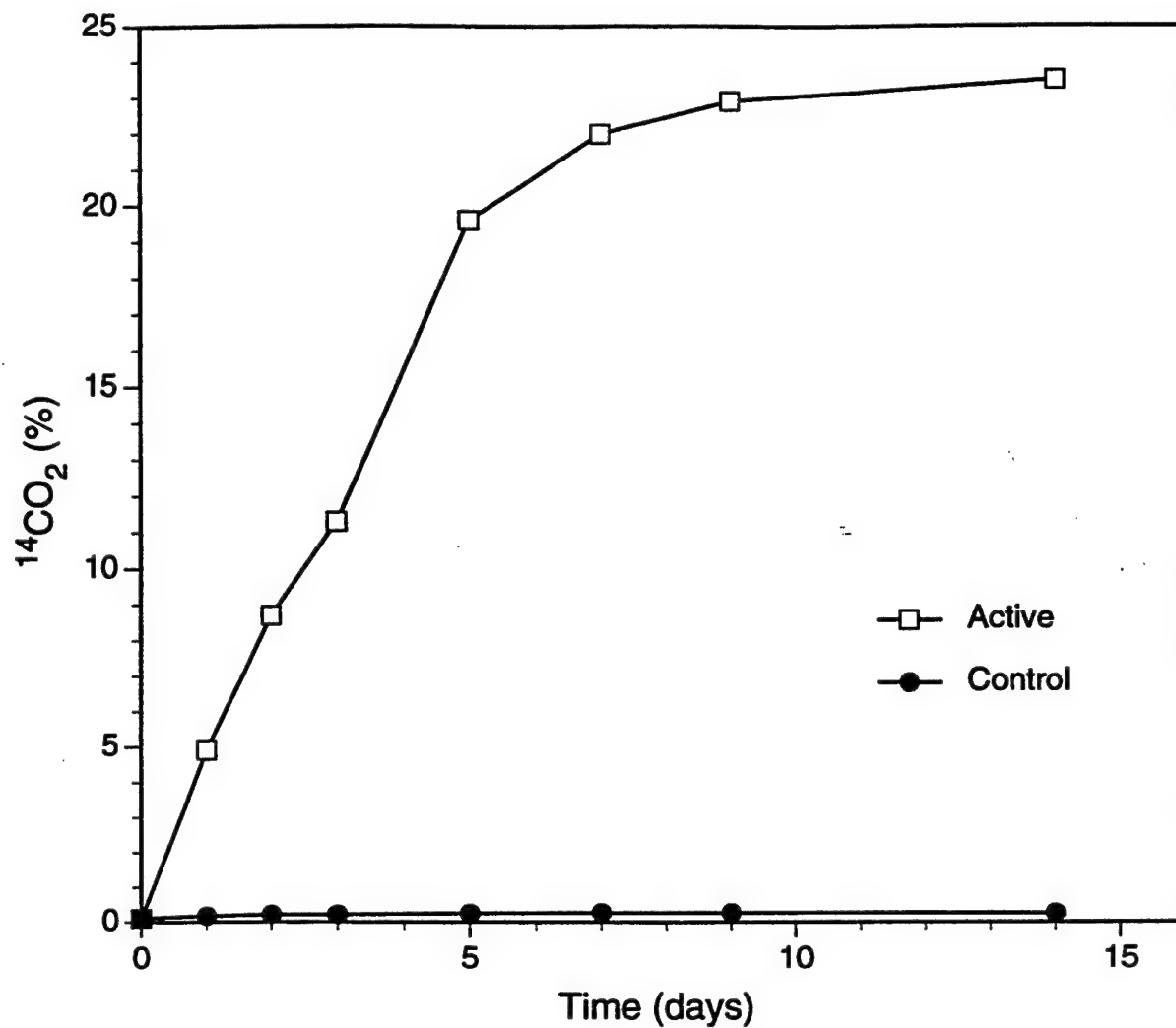FIGURE 36 pH and Oxygen Uptake Rate in the Reactor

FIGURE 37 $^{14}CO_2$ Produced by the Reactor Biomass

amount of [^{14}C]TNT (27%) was observed as TCA-precipitable material on day 14. In the killed control, the TCA-precipitable material observed was 0.4% (Figure 38). This experiment indicated that the biomass in the slurry reactor actively used the [^{14}C]TNT to make cellular materials. Figure 39 shows the distribution of radioactive carbon after 30 days of incubation. This distribution is the same as that on day 14.

The radiolabeling studies on the reactor biomass revealed various intermediates that had been identified before and another previously unidentified intermediate that eluted at about 2.2 min (Figure 40). The identified intermediates were 4-amino-2,6-dinitrotoluene, 2-amino-4,6-dinitrotoluene, and 2,4-diamino-6-nitrotoluene. Similar intermediates have been observed in the slurry reactors.

The gas chromatogram of a reactor sample showed two distinct peaks that eluted at about 5.8 and 6 min (Figure 41). Mass spectral analysis of the peak eluting at 6 min showed a fragmentation profile similar to that of 2,3-butanediol (Figure 42). The other peak showed similarity to the isomer, 1,3-butanediol. Standard 2,3-butanediol eluted at about 6 min (Figure 43). The mass spectrum of standard 2,3-butanediol exactly matched that of the sample peak that eluted at 6 min.

The major ions present in 2,3-butanediol are 45, 57, and 75. These ions matched perfectly in the standard 2,3-butanediol and in the peak in the reactor sample that eluted at 6 min (Figure 44), confirming that the intermediate produced during TNT metabolism was 2,3-butanediol. To determine whether 2,3-butanediol is present in the molasses serving as the main substrate in the reactors, we analyzed molasses by GC/MS. The chromatogram for molasses had several peaks (Figure 45), but none matched the mass spectrum of the standard 2,3-butanediol. This result confirmed that the intermediate 2,3-butanediol was the sole product of TNT metabolism by the soil bacteria.

Analysis of nitrite and ammonia in the soil slurry showed that no nitrite was produced in the reactor and that the concentration of ammonia varied from 500 to 950 mg/L throughout the study (Figure 46). After 200 days of reactor operation the feeding schedule was changed, and the time course of the reactor operation was reset to day 0. Of the two reactors, one received soil and carbon additions three times weekly, and the other received soil and carbon additions three times weekly. The operation of the reactors under the new feeding schedule was continued for 50 days. The results showed that increasing the frequency of soil addition did not affect the efficiency of TNT removal (Figure 47). However, accumulation of the 2-amino intermediate to about 200 mg/kg of soil was observed in the reactor that received soil additions three times weekly. The pH and oxygen uptake were greatly affected by the increase in the frequency of soil addition. As Figure 48 shows, the pH was highly acidic (4-5) in the reactor that received soil three times per week, requiring constant adjustment. The oxygen uptake rate also decreased to 4 mg/L in the reactor that received soil three times weekly (Figure 49). This result indicates that the biomass in the reactor was affected by the accumulation of either TNT metabolites or molasses metabolites.
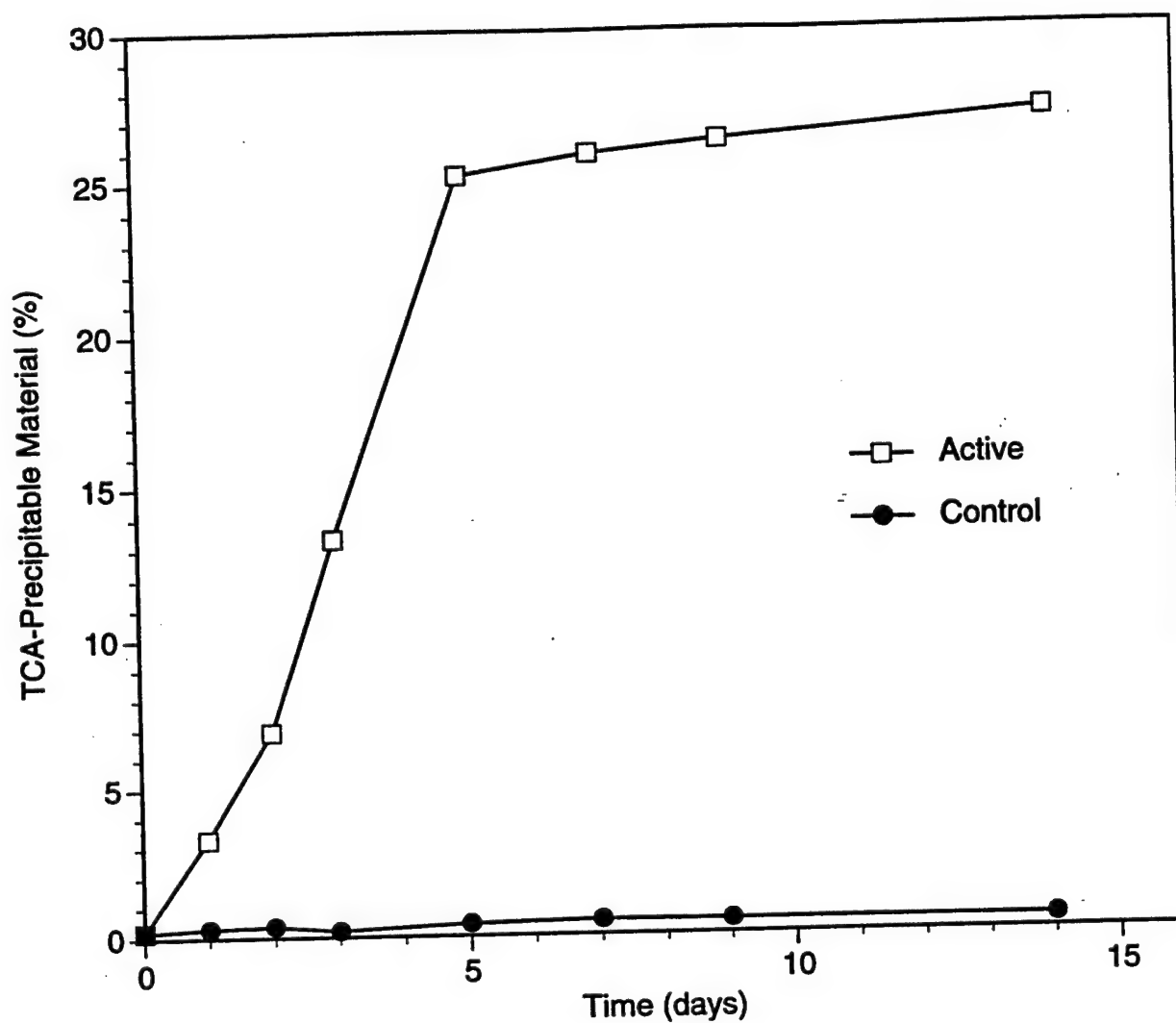
FIGURE 38   TCA-Precipitable Material in the Reactor Biomass
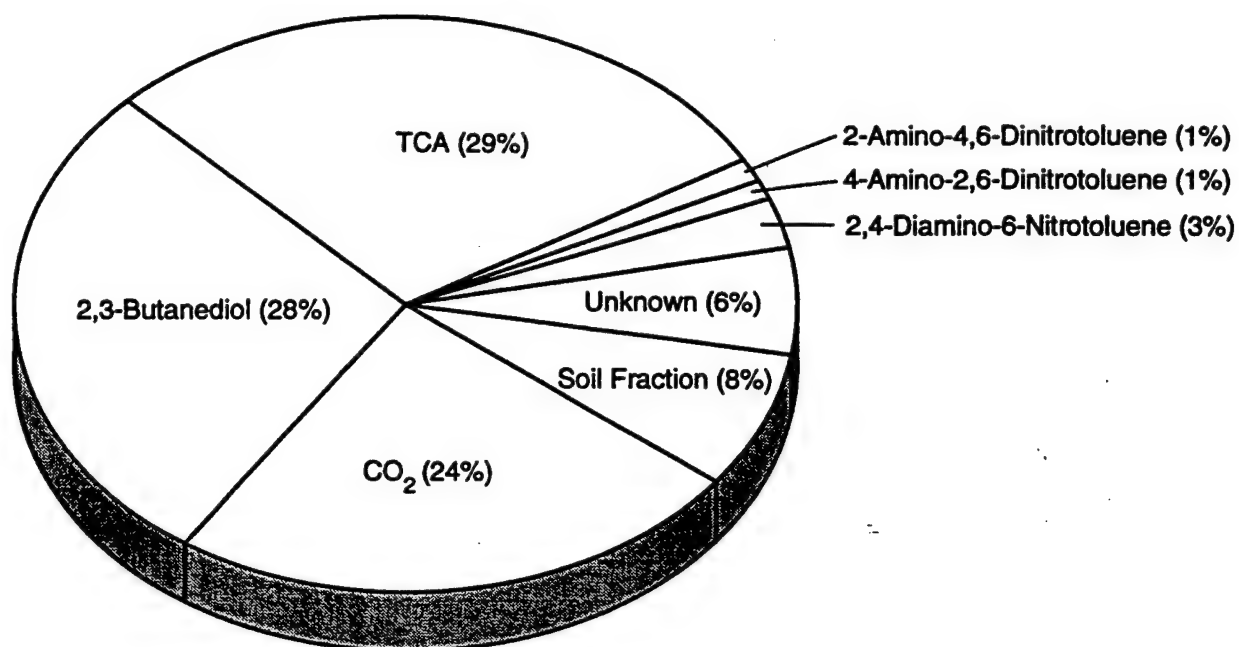
FIGURE 39   Distribution of Radiolabel after 30 Days (Average Recovery 90-95%)
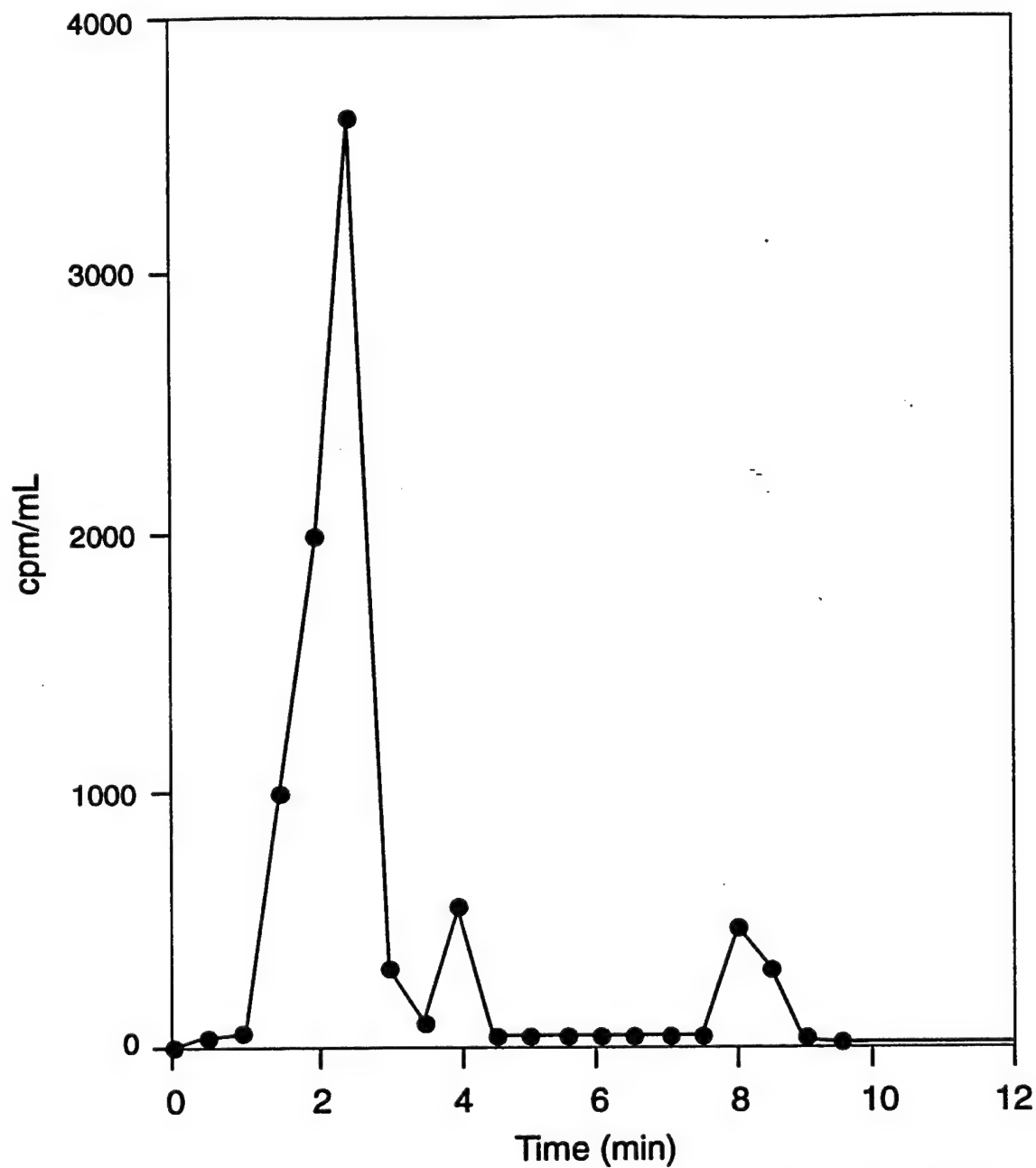
FIGURE 40 Radiometric HPLC Analysis on Day 14 after the Addition of [^{14}C]TNT
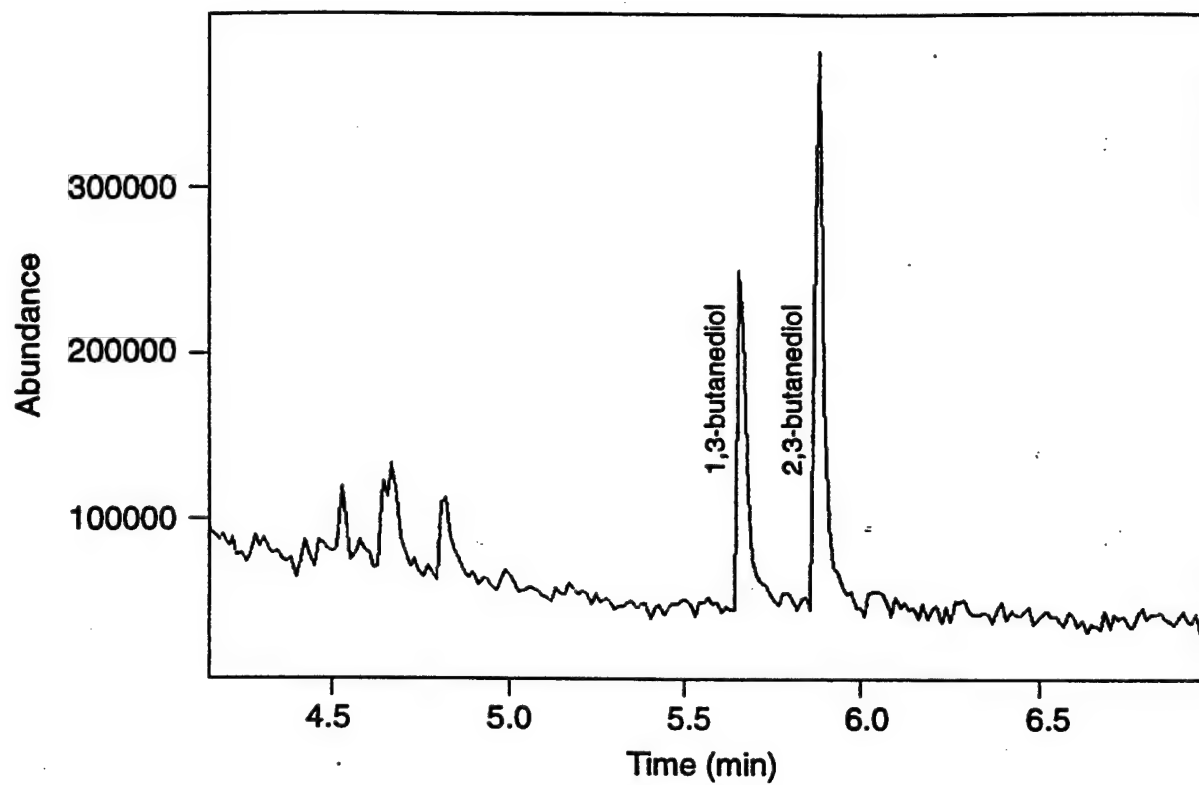
FIGURE 41 GC/MS Chromatogram of 2-min HPLC Peak in Figure 40
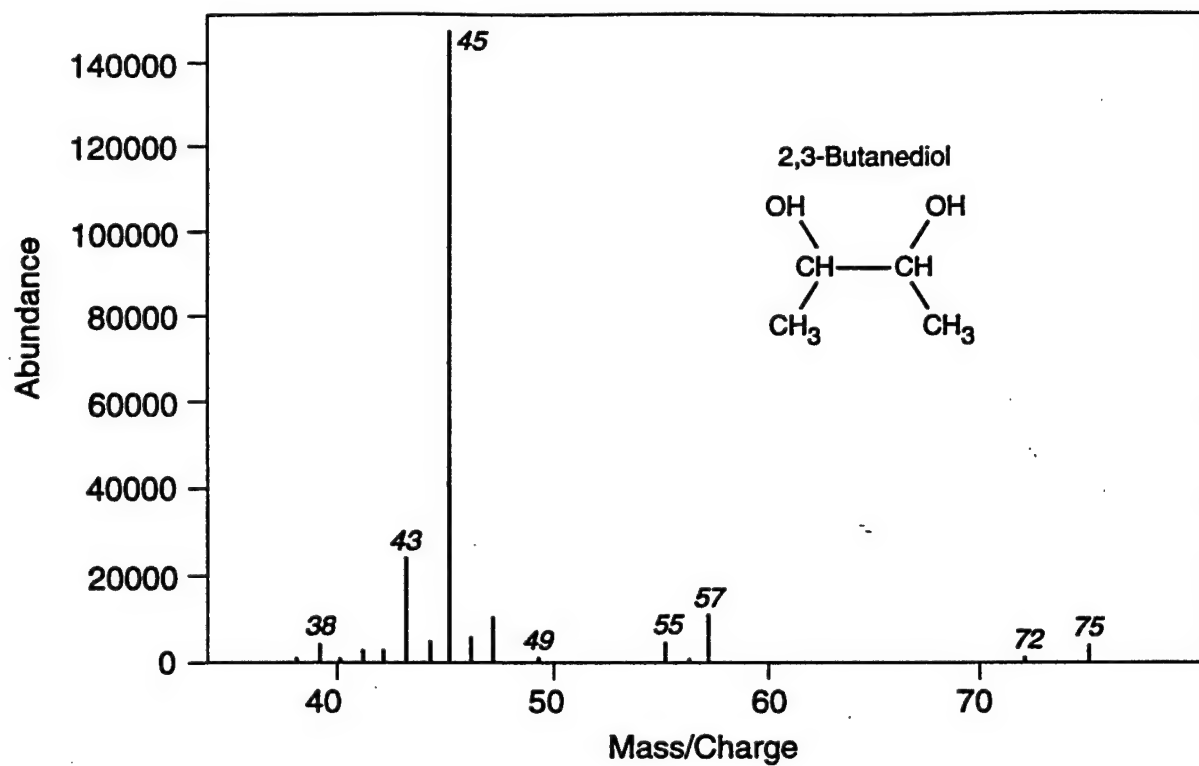
FIGURE 42 Mass Spectrometric Pattern of 6-min GC/MS Peak in Figure 41
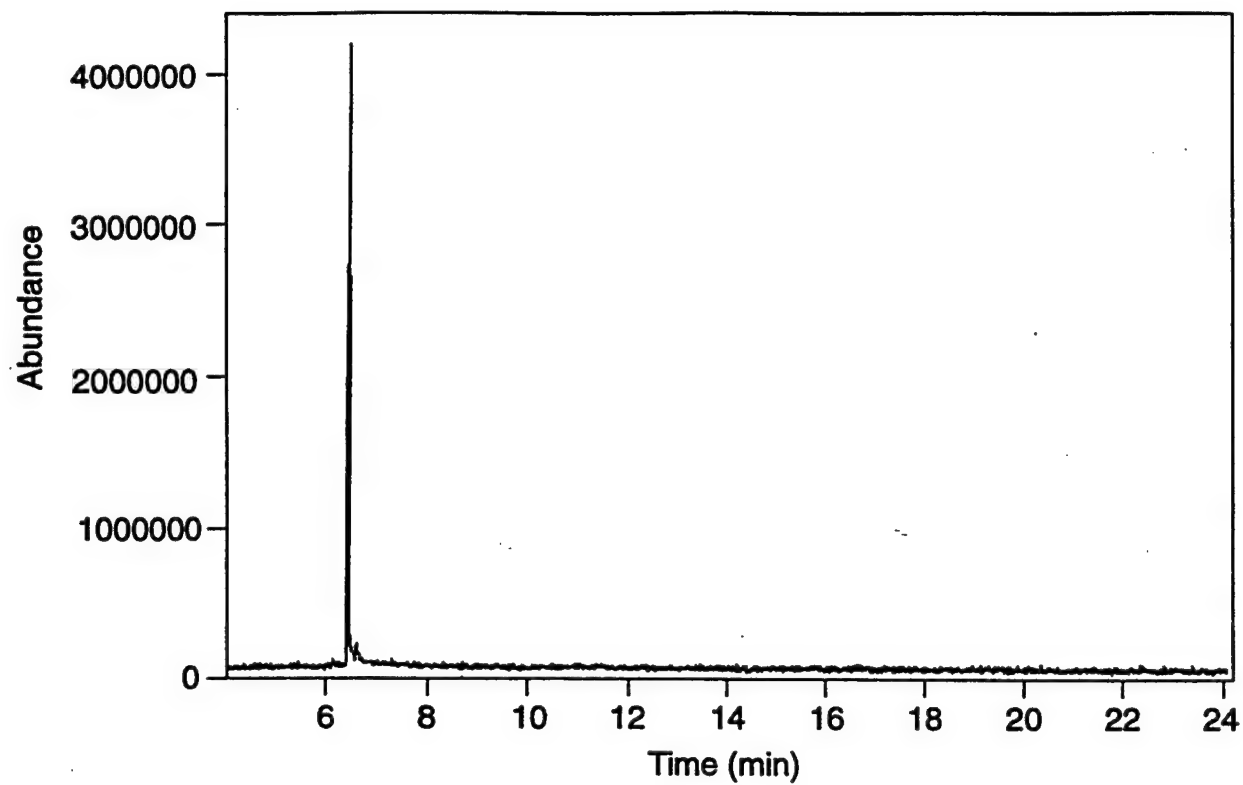
FIGURE 43 GC/MS Chromatogram of 2,3-Butanediol Standard
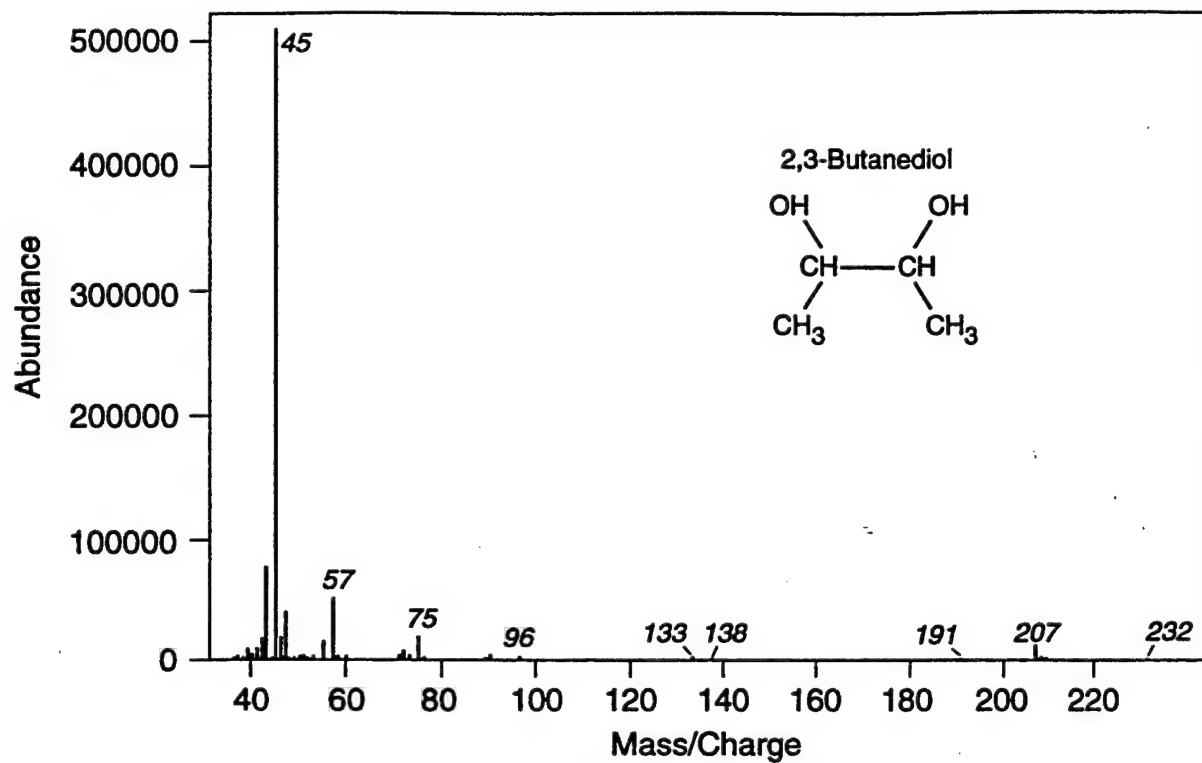
FIGURE 44   Mass Spectrometric Fragmentation Pattern of 2,3-Butanediol
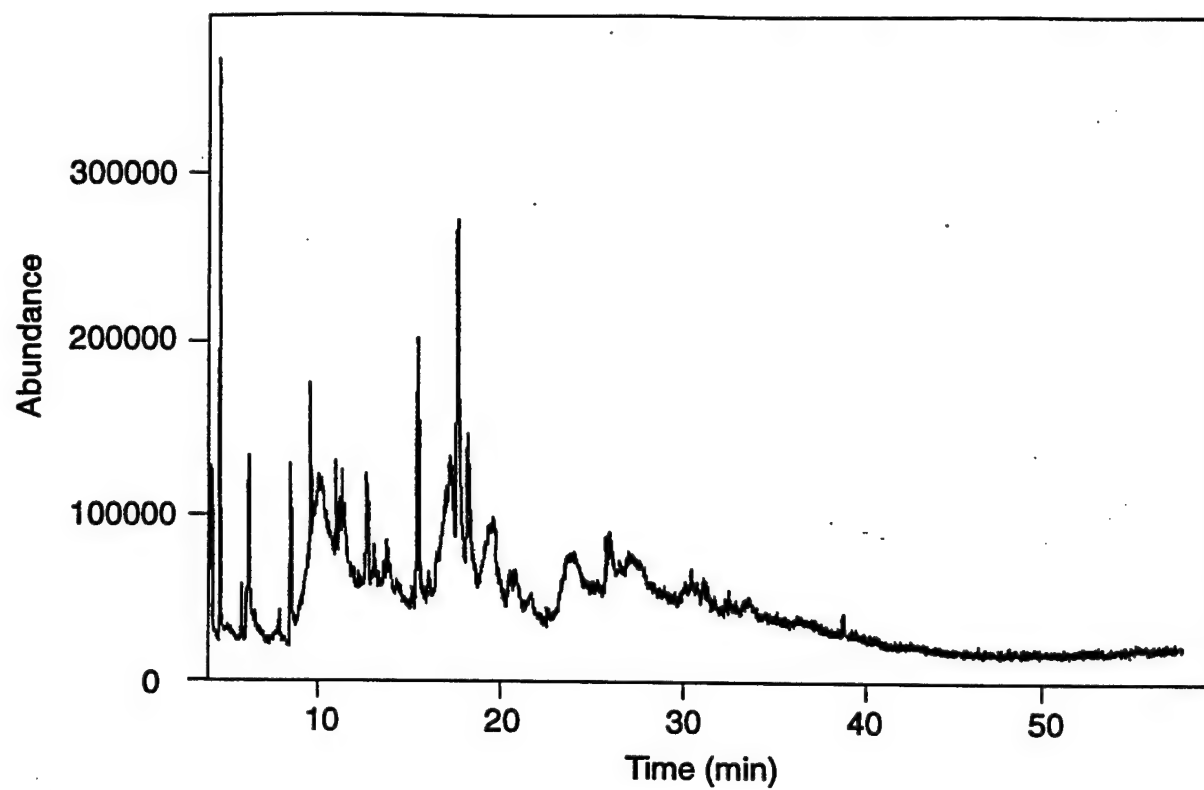
FIGURE 45 GC/MS Chromatogram of 0.3% Molasses
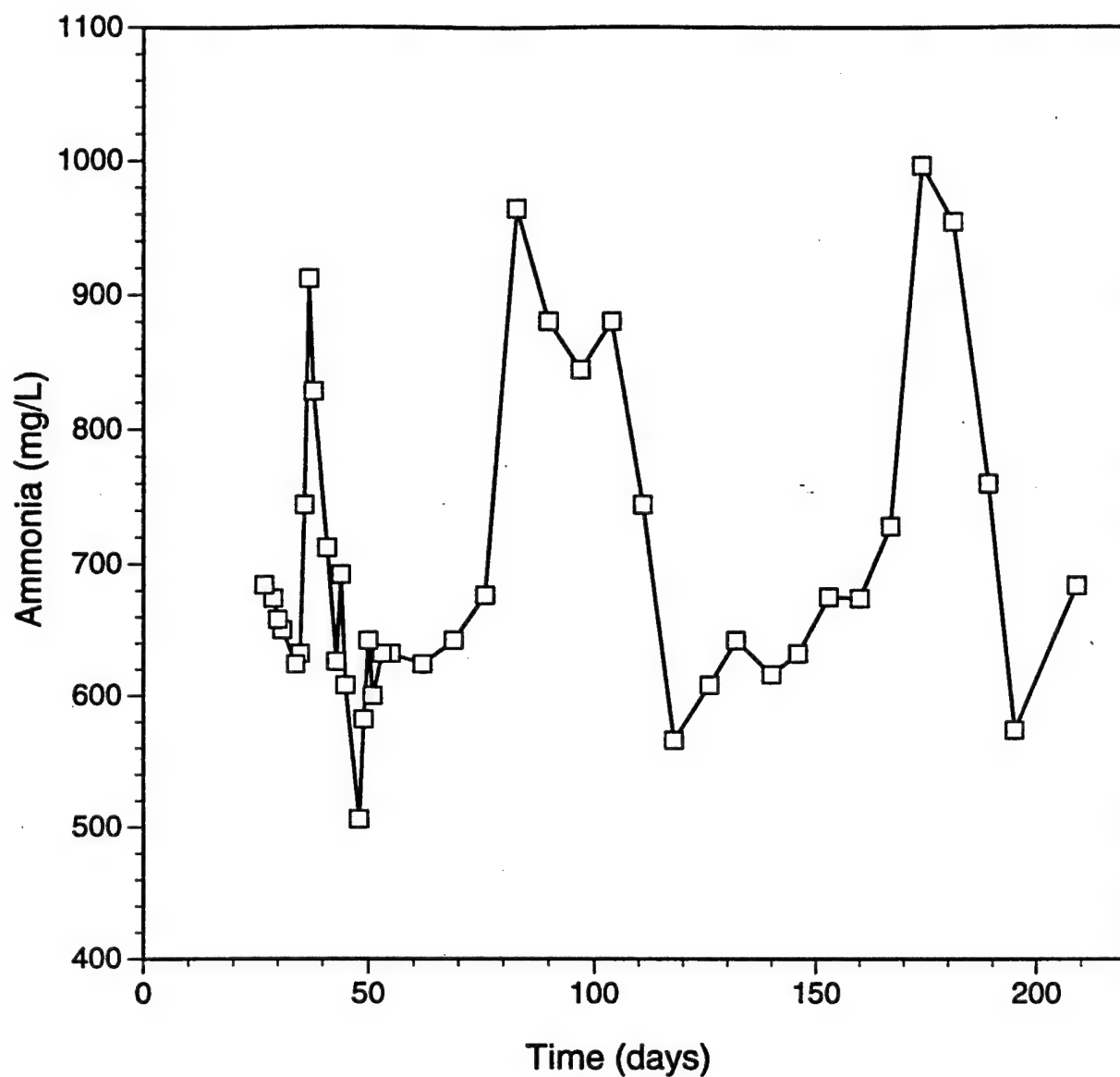
FIGURE 46 Ammonia Concentration in the Reactor
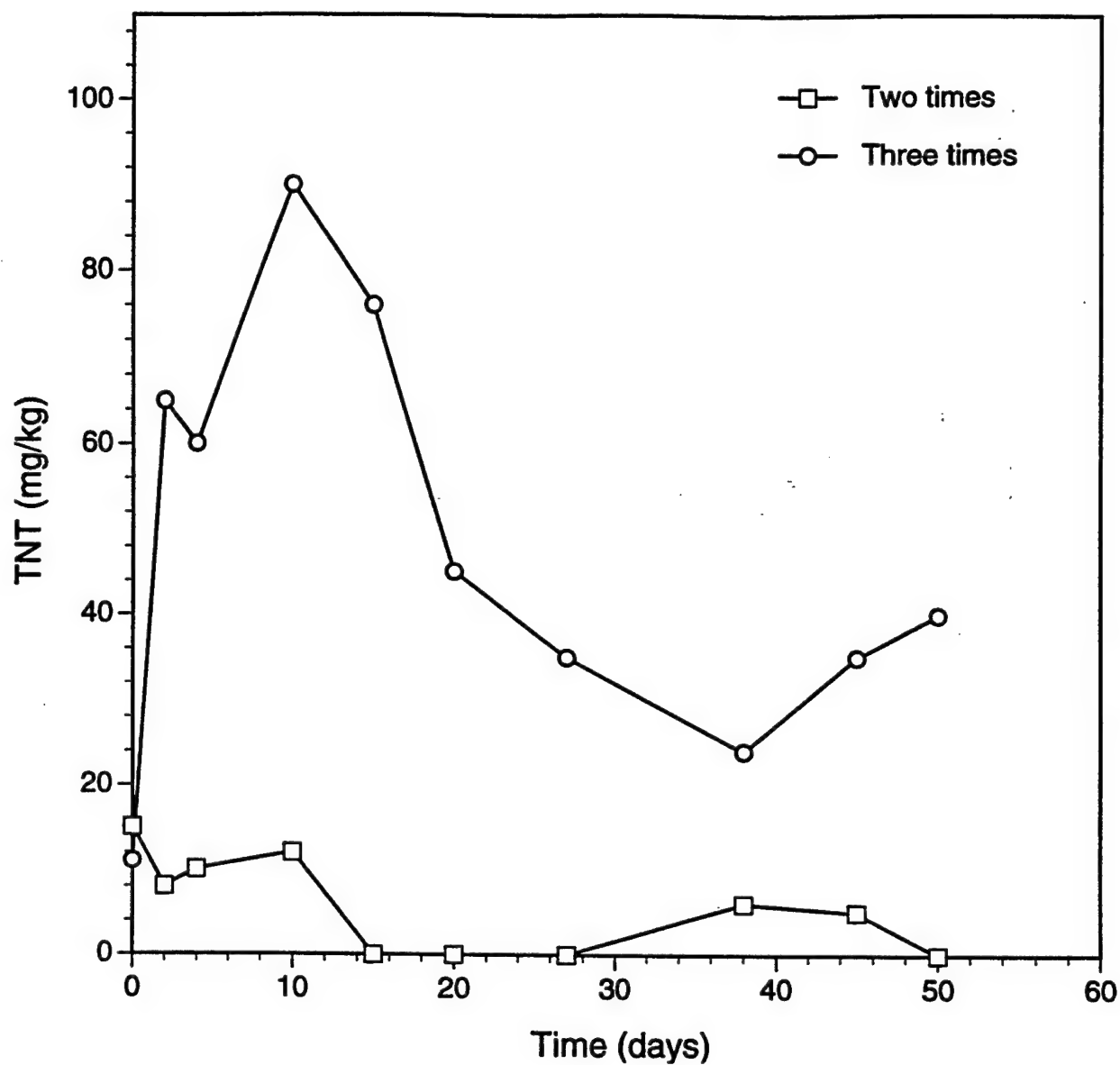
FIGURE 47 TNT Concentration in Reactors Fed Two or Three Times Weekly

FIGURE 48 pH in the Reactors with More Frequent Soil Feedings

FIGURE 49 Oxygen Uptake Rates in the Reactors with More Frequent Soil Feedings

This study showed that the aerobic/anoxic soil slurry reactor is an effective method for remediating TNT and other munitions compounds present in the contaminated soil. The operation of laboratory-scale soil slurry reactors over 200 days showed that 100% removal of TNT can be achieved. During startup, a two-week stabilization period was needed before further loading of soil into the reactor.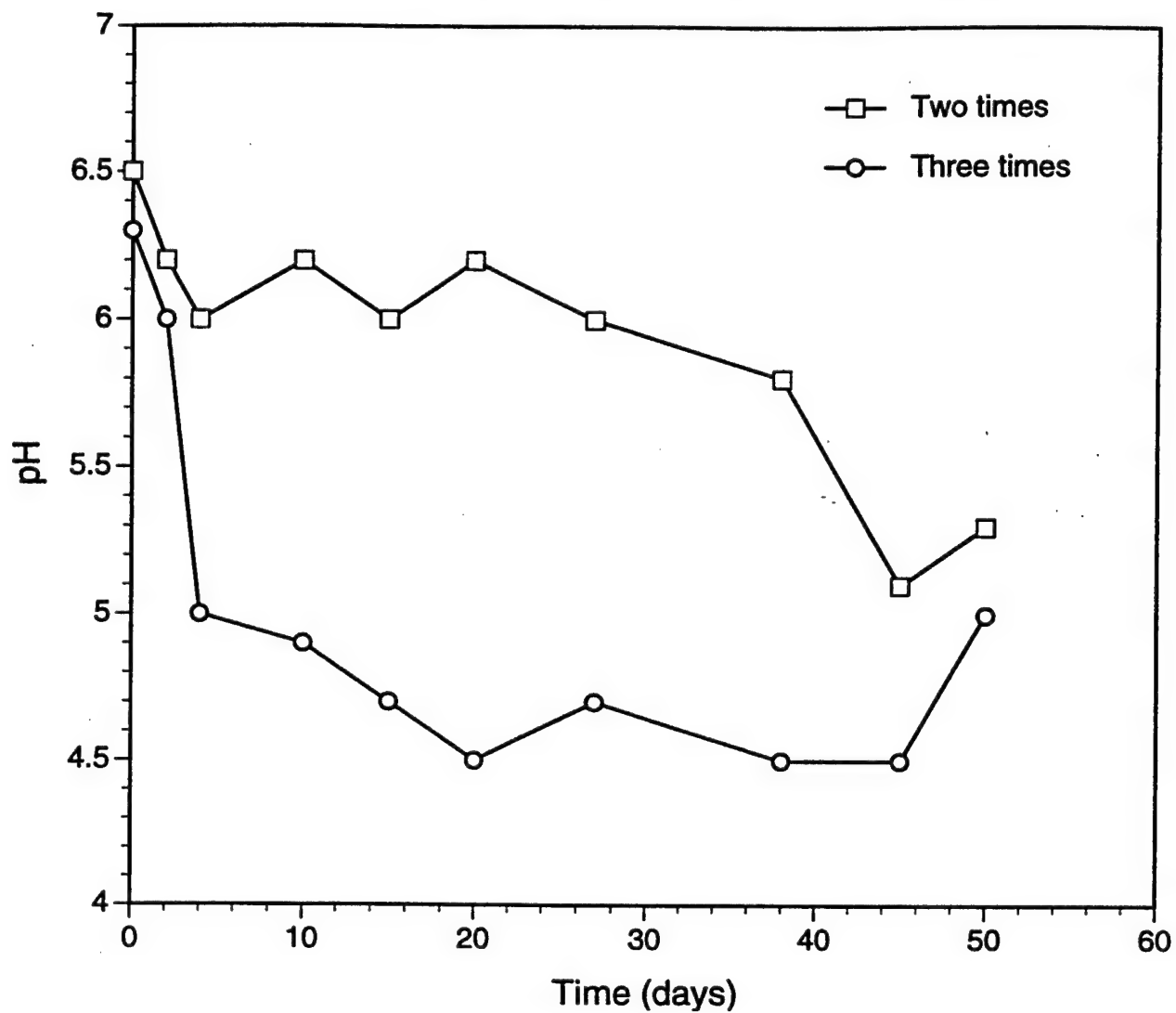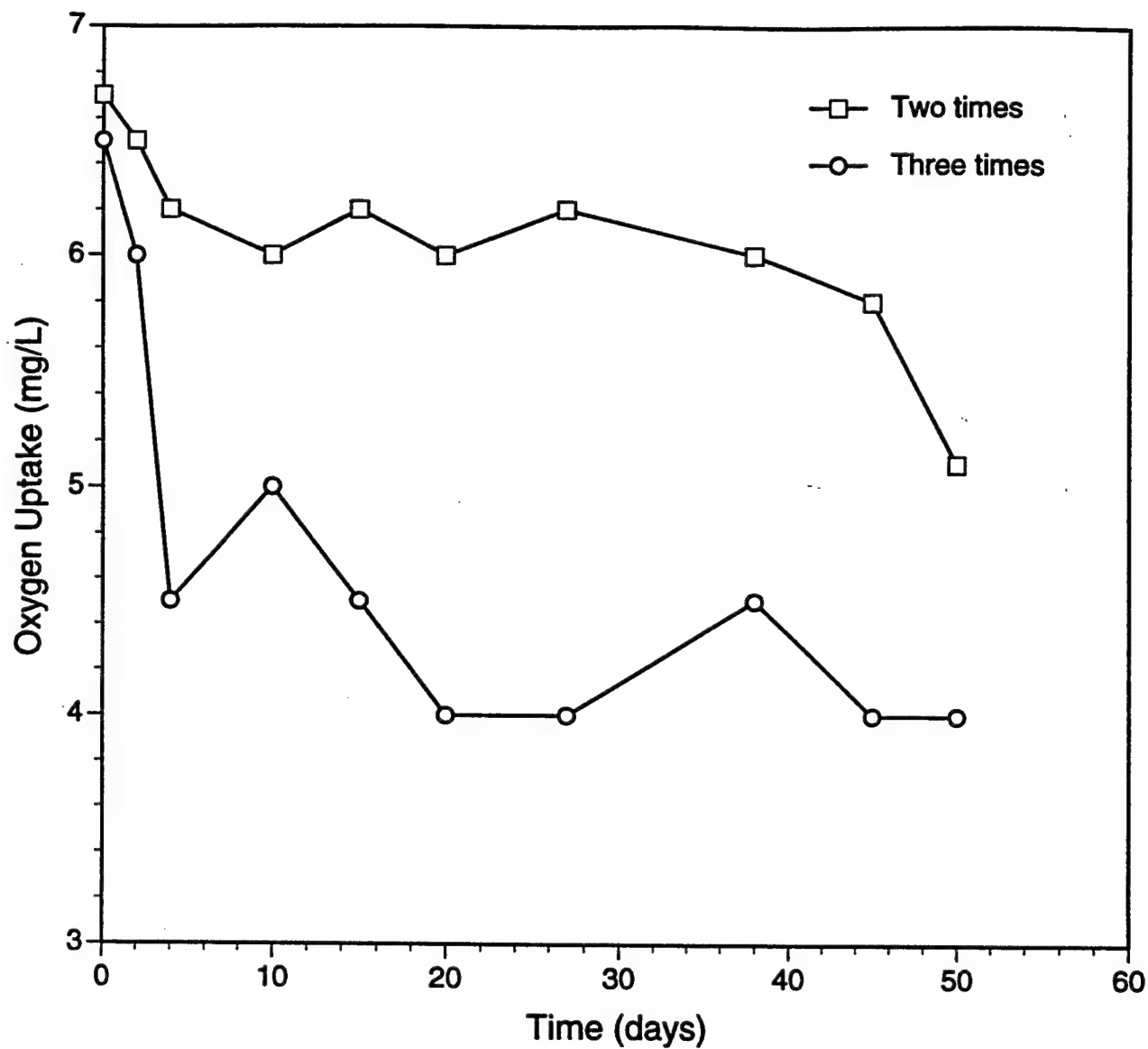 Operation of the reactor was highly successful in the semicontinuous mode. TNT was also removed in the batch mode of operation (during the first two weeks of operation). However, TNT metabolites (4-amino and 2-amino compounds) persisted during batch operation. For successful removal of TNT and its metabolites, semicontinuous operation is recommended. Throughout the study no reactor failure occurred because of overloading or the accumulation of toxic metabolites.

The radiolabeling study showed a very reasonable mass balance of [^{14}C]TNT. Of the [^{14}C]TNT (20,000 cpm/mL) introduced at the beginning of the experiment, 23% was converted to $^{14}CO_2$, and 27% was used to make cellular materials. Extraction of soil with acetonitrile showed that 8% of the TNT was adsorbed to the soil. The rest of the TNT was accounted for as intermediates. The major intermediates were 4-amino-2,6-dinitrotoluene (1.5-2.5%), 2-amino-4,6-dinitrotoluene (1.6-3.0%), 2,4-diamino-6–nitrotoluene (3.0%), and an unidentified intermediate (30%) that eluted at 2.1 min under the chromatographic conditions used (Table 4). The mass balance was very reasonable, with recovery of 95% of the [^{14}C]TNT. Continued incubation of the slurry for 60 days resulted in complete removal of intermediates, except for the unidentified intermediate, which remained in the slurry at 19-20%. The addition of molasses did not help to remove this intermediate.

This study showed that the natural soil bacteria present in contaminated soil can cause extensive transformation and degradation of TNT in a reasonable time under optimal conditions. Degradation was demonstrated by mineralization of [^{14}C]TNT, formation of metabolites, and the presence of [^{14}C]TNT in the cell biomass as TCA-precipitable material. Each of the biological systems reported in the literature as acting on TNT catalyzed the reduction of at least one nitro group (Won et al. 1974; Schackmann and Muller 1991). In the present study the microbes produced amino intermediates and also were able to mineralize TNT significantly. The radiolabeling experiments showed that the biomass from prolonged reactor operation (over 200 days) converted [^{14}C]TNT to $^{14}CO_2$. Because the ring carbons of TNT were uniformly labeled, conversion to CO_2 clearly denotes ring cleavage. The mechanism of TNT ring cleavage is not clear. However, the first step was the reduction process reported before by many workers (McCormick et al. 1976; Schackmann and Muller 1991; Won et al. 1974).

Among the different bioremediation methods reported for munitions compounds, the soil slurry reactor system seems to be very promising. The composting method described by Williams et al. (1992) removes TNT, RDX, and HMX under thermophilic and mesophilic conditions. The disadvantage of composting is that it needs a large quantity of additives and results in remediation of a low volume of contaminated soil. The soil slurry reactor system in our study successfully

TABLE 4 Mass Balance of Radioactive TNT In the Reactor Biomass

	$^{14}CO_2$	TCA-Precipitable	Soil Fraction	Distribution (%)[a] 4-Amino[b]	2-Amino[c]	2,4-Diamino[d]	2,3-Butanediol	Total Recovery
Reactor 1	22.9	27.2	7.8	2.5	1.6	3.0	30	95
Reactor 2	23.9	27.7	8.1	1.5	3.0	3.3	28.3	95.8

[a] Data were collected after 14 days of incubation.

[b] 4-Amino-2,6-dinitrotoluene.

[c] 2-Amino-4,6-dinitrotoluene.

[d] 2,4-Diamino-6-nitrotoluene.

removed TNT, RDX, HMX, and TNB and other contaminants present in the soil. The advantage of the slurry reactor is its simplicity of operation. The method needs only mixing, an intermittent supply of air, and a carbon source. Molasses is an inexpensive carbon source that could be used in a large-scale operation at low cost. The frequency of soil replacement in the system can be determined on a site-specific basis, depending on the concentrations of TNT in the contaminated soil.

6 Summary and Conclusions

This study demonstrated that a soil bacterial consortium isolated from TNT-contaminated soil can cause extensive transformation of TNT in a reasonably short period of time. The TNT transformation was accomplished by co-metabolism. Among the different substrates studied, the relatively inexpensive molasses was found to produce the best result in terms of bacterial growth and TNT transformation. Evaluation of environmental parameters revealed that a pH of 7 and a temperature of 22-24°C are optimal for bacterial activity. Nitrogen is an important factor in bacterial growth. The bacteria were unable to use TNT as a nitrogen source, and nitrogen in the form of ammonium chloride had to be supplemented for bacterial growth and TNT metabolism. The optimization studies showed that 0.5 g/L of ammonium chloride as a nitrogen source was ideal for TNT metabolism.

The HPLC and GC/MS analyses of culture samples identified the two main intermediates as 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene. The radiolabeling study with the aerobic/anoxic reactor biomass indicated that 22-23% of TNT was mineralized to CO_2, 27% was converted to biomass or a TCA-precipitable fraction, and the rest became TNT metabolites (including 30% as an unidentified intermediate). The mass balance was reasonably accurate.

The aerobic bacterial consortium was unable to grow under anoxic, nitrate-reducing conditions. The consortium used molecular oxygen as its electron acceptor. Analysis for cell-bound TNT showed that TNT was slightly adsorbed to the cell walls of bacteria. TNT was removed not only by growing cells but also by resting cells and cell-free extracts. The continuous addition of TNT to the culture medium did not inhibit bacterial growth or TNT removal by the bacterial consortium. The induction studies using purine and pyrimidine bases revealed that TNT metabolism was not induced by these nitrogenous compounds.

Four *Pseudomonas* spp. were isolated from the consortium. The experiments conducted with individual *Pseudomonas* spp. suggested that each species has a specific function in TNT metabolism by the consortium. All four species metabolized TNT.

The laboratory-scale aerobic/anoxic soil slurry reactor study showed that TNT can be removed and mineralized by native soil bacteria. Molasses was the best substrate. The soil slurry reactors were operated successfully for a period of eight months. The bacterial population not only tolerated the high concentration of TNT in the soil but successfully removed it. The bacterial population was maintained at about 10^8 CFU/mL in the reactor. The slurry reactor also removed the munitions compounds RDX, HMX, TNB, and DNT. The cost of molasses is significantly less than the cost of succinate and other substrates; hence, its use in a large-scale biotreatment system may make such soil treatment economically viable.

7   References

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